Investigation of myostatin and relevant regulators during muscle regeneration after an acute bout of eccentric exercise

by
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Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of Nature Sciences at Stellenbosch University

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December 2014

Declaration

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Johannes David Conradie *Date*

December 2014

Abstract

The aim of this study was to investigate the powerful muscle regulator, myostatin, and its regulators in response to an acute bout of plyometric training. The participants were recruited and screened by characterization by means of isometric force production tests, baseline blood creatine kinase levels and $VO_{2\ max}$ results. The selected individuals (n=15) were subjected to a baseline muscle biopsy for comparative purposes. The study made use of plyometric jumping, as source of eccentric exercise, to serve as an exercise intervention after which muscle biopsies (4 hours post and 24 hours post) and blood draw (4 hours post, 24 hours post and 48 hours post) samples were taken. Maximal voluntary isometric contractions of the knee extensors were also measured immediately after the exercise protocol and after 1 week recovery. Creatine kinase (CK) analysis on the serum samples was used to conclude muscle damage. The muscle biopsy samples were used for protein quantification (Western blot) and gene expression assessment (semi-quantitative and real-time PCR). The results showed decreased force production immediately after eccentric exercise ($p < 0.05$), while returning back to baseline values at 1 week post exercise and CK results showed a significant increases at 4 hours ($p<0.05$), 24 hours ($p<0.001$) and 48 hours ($p<0.01$) after exercise. There were no significant differences in myostatin precursor protein (43 kDa), phosphorylated Smad2,3, Smad7 or activin receptor IIb in response to eccentric exercise. However, the follistatin protein was increased at both 4 hours and 24 hours after exercise ($p<0.01$). RNA analysis of the extracellular matrix (ECM) protein, decorin, revealed the existence of the splice variants A1 and A2 in human skeletal muscle. The RT-PCR analysis (n=4) of these variants showed no significant difference when comparing pre- to post-exercise. The decorin core protein was also investigated by means of antibody probing and results revealed the need for ABC chondroitinase enzyme treatment before immunoblotting of human skeletal muscle samples. The results concerning knee extensor force reduction and circulating creatine kinase showed the effectiveness of plyometric jumping in producing skeletal muscle damage in the lower limbs of unfit individuals, unaccustomed to eccentric exercise. In conclusion, myostatin, and its associated signalling cascade, are not activated in early muscle regeneration, but follistatin is increased during this phase possibly aiding and initiating the muscle repair process. Future studies: Variants of decorin are expressed in human skeletal muscle, increasing the complexity that should be taken into account in studies concerning the regulation of decorin in a human model. Investigation into myostatin protein at different post-translational levels needs more clarification. Published methods and materials used in different laboratories are not consistent and investigators should attempt to standardise protocols in order to compare results between studies more effectively. Of importance, these results show that the myostatin at protein level report different results compared to mRNA analysis and that more investigation into myostatin regulatory factors, with special reference to follistatin and decorin, is needed in future human models.

Opsomming

Die doel van hierdie studie was om die kragtige spiere reguleerder, miostatin, en sy reguleerders in reaksie op 'n akute aanval van pliometriese spronge te ondersoek. Die deelnemers is gewerf en gekeur deur karakterisering deur middel van isometriese krag produksie toetse, basislyn bloed kreatien kinase vlakke en VO_2maks resultate. Die geselekteerde individue (N = 15) is onderhewig aan 'n basislyn spierbiopsie vir vergelykende doeleindes. Die studie het gebruik gemaak van pliometriese spronge (essentriese spier aksie) as die oefening intervensie waarna spierbiopsie (4 uur na en 24 uur na) en bloed (4 uur na, 24 uur na en 48 uur na) monsters geneem is. Isometriese kontraksies van die knieverlengers is ook gemeet onmiddellik na die oefening protokol en na 1 week se herstel. Kreatine kinase (KK) ontleding van die serum monsters is gebruik om spierskade aftelei. Die spierbiopsie monsters was gebruik vir proteïen kwantifisering (Western klad) en die assessering van geen uitdrukking (semi-kwantitatiewe en real-time PCR). Die resultate het gewys dat krag produksie afgeneem het onmiddellik na essentriese oefening ($p < 0.05$), terwyl dit terugkeer na die oorspronklike waardes 1 week na oefening en KK resultate toon 'n beduidende toename by 4 uur ($p < 0,05$), 24 uur ($p < 0,001$) en 48 uur ($p < 0,01$) na oefening. Daar was geen betekenisvolle verskille in Miostatien voorloper proteïen (43 kDa), gefosforileerde Smad2,3, Smad7 of Activin reseptoor IIb in reaksie op essentriese oefening. Dit is egter die follistatien proteïen wat verhoog by beide 4 uur en 24 uur na oefening ($p < 0,01$). RNS ontleding van die ekstrasellulêre matriks (ESM) proteïen, decorin, het die bestaan van die splitsing variante A1 en A2 in menslike skeletspier, aan die lig gebring. Die RT-PCR analise (n = 4) van hierdie variante het geen betekenisvolle verskille getoon wanneer voor met na-oefening vergelyk is. Die decorin kern proteïen is ook ondersoek deur middel van teenliggaam afhanklike metodes en resultate het die behoefte aan ABC chondroitinase ensiem behandeling voor immunokladding van menslike skeletspier monsters gesteun. Die resultate aangaande knieverlenger krag vermindering en sirkuleerende kreatien kinase het die doeltreffendheid van pliometriese spronge in die vervaardiging van skeletspier skade in die onderste ledemate van individue ongewoond aan essentriese oefening verseker. Ten slotte, Miostatien, en sy verwante sein kaskade, is nie geaktiveer vroeg in spier herstelling, maar follistatien is tydens hierdie fase verhoog en help moontlik met die aanvang van die spier herstel. Toekomstige studies: variante van decorin word uitgedruk in menslike skeletspier, wat die kompleksiteit aangaande decorin verhoog en dit is iets wat in ag geneem moet word in studies wat handel oor die regulering van decorin in mens modelle. Ondersoek na miostatien proteïen op verskillende na-translasie vlakke moet meer duidelikheid verkry. Gepubliseer metodes en materiaal wat gebruik word in verskillende laboratoriums is nie konsekwent en ondersoekbeamptes moet probeer om protokolle te standaardiseer sodat resultate van studies meer effektief kan vergelyk word. Van belang is, die resultate wys dat miostatien op proteïen vlak verskillende resultate vertoon in vergelyking met boodskapper-RNS ontleding en dat

meer ondersoek na miostatien regulerende faktore, met spesiale verwysing na follistatien en decorin, nodig is in toekomstige menslike modelle.

Acknowledgements

God, for giving me the potential to succeed in any and all quests

I can do all this through him who gives me strength.(Philippians 4:13)

My parents, family and friends for support and understanding

De Witt family, for support and motivation throughout my studies

Professor K.H. Myburgh for guidance and the opportunity to complete an MSc

Dr. P.J Durcan for assistance and guidance in completion of my thesis

Paul Steyn for assistance in the laboratory

Prof. Kidd for assistance with statistical analysis

Central Analytical Facility for assistance with genetic analysis

The department of Physiological sciences for a welcoming and energetic working environment

National Research Foundation (NRF) for financial support

Dedication

This thesis is dedicated, in a big part, in memory of Benjamin Fredeman Calitz / Oupa Ben (11/02/1926 – 14/08/2012), who provided me with personal and financial support throughout my studies. He inspired me and motivated me to work towards a better and/or higher education.

List of Abbreviations

1RM – 1 Repetition maximum

aa – Amino acid/s

Ach – Acetylcholine

ActRIIB - Activin receptor type IIb

ADP – Adenosine diphosphate

AE – Aerobic exercise

AGAT - Arginine:glycine amidinotransferase

AIDS – Acute immunodeficiency syndrome

ALK – Anaplastic lymphoma kinase

AMP – Adenosine monophosphate

ATP - Adenosine triphosphate

bHLH – Basic helix-loop-helix

BMP – Bone Morphogenetic protein

cDNA – complementary Deoxyribonucleic acid

CE – Concentric exercise

CK – Creatine kinase

DNA - Deoxyribonucleic acid

DOMS – Delayed onset muscle soreness

ECM – Extracellular matrix

EE – Eccentric exercise

ELISA – Enzyme linked-immunosorbent assay

F – Female

FGF – Fibroblast growth factor

FoxO – Forkhead box O

FST – Follistatin

FSTL – Follistatin-like

GAG – Glycosaminoglycans

GAMT - Guanidinoacetate methyltransferase

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GDF – Growth differentiation factor

GSK3β - Glycogen synthase kinase 3-beta

HGF – Hepatocyte growth factor

Ig – Immunoglobulin

IGF – Insulin-like growth factor

IGFBP – Insulin-like growth factor binding protein

IRS-1 – Insulin receptor substrate-1

Kd – dissociation constant

LDH – Lactate dehydrogenase

M – Male

MAT - Methionine adenosyltransferase

MGF – Mechano growth factor (also known as, IGF-1Ec)

MRF – Myogenic regulatory factor

mRNA – messenger Ribonucleic acid

MSTN – Myostatin/GDF-8

Mt – Mitochondria

mTOR – Mammalian target of rapamycin

MVC – Maximal voluntary contraction

PCR – Polymerase chain reaction

PI3K – Phosphoinositide 3-kinase

RE – Resistance exercise

RT – Resistance training

SLRP – Small leucine rich proteins

SR – Sarcoplasmic reticulum

TAD - transcriptional activation domain

TGF – Transforming growth factor

TLD – Tolloid

Tm – melting temperature

TSC 1/2 - Tuberous sclerosis complex-1 and 2

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1 Chapter 1: Introduction

1.1 Structure of skeletal muscle

The skeletal muscle system is responsible for movement by shortening the sarcomeres and producing force. Skeletal muscle accounts for approximately 40% of total body weight in men and 32% in women thus making it the heaviest organ of the human body. Unlike smooth and cardiac muscle cells, skeletal muscle is considered to be striated and voluntary (1).

Skeletal muscle is made up of a numerous multinucleated muscle fibres, positioned parallel to each other, and bundled together by connective tissue. During embryonic development muscle fibres are constructed by the fusion of mononucleated myoblasts. Within each muscle fibre, the major structural feature is the existence of multiple myofibrils (see Figure 1.1 for schematic of a myofibril). The myofibrils are made up out of thick (myosin) and thin (actin) filaments. When myofibrils are investigated with electron microscopy, a parallel pattern of dark (A bands) bands and light (I bands) are observed. The A-bands, consist of thick filaments and have a lighter area in the middle, known as the H-zone. The M-band runs down the middle of the H-zone in the centre of the sarcomere and is important to hold the thick filaments together vertically. The I-band is made up of thin filaments and in the middle of the I-bands is a dense line called the Z-disk. The Z-disks are known as the borders of the sarcomeres, which are the functional units of skeletal muscle. The Z-disk plays an important role in structural stability during contraction and links the sarcomeres to other elements of the intracellular cytoskeleton e.g. desmin. (1)

Titin is another important part of the sarcomere structure as it overlaps the Z-disk and M-band of the sarcomeres resulting in a continuous elastic filament within the cell (2). Multiple structural domains are present in Titin including the I-band domain of titin that is made up of Immunoglobulin-like (Ig)-domains and PEVK and N2B segments, which affect elasticity and are activated by muscle stretching (3–5). Affected titin expression has been found to possibly lead to severe myopathies and premature death, emphasizing the important of titin (6,7).

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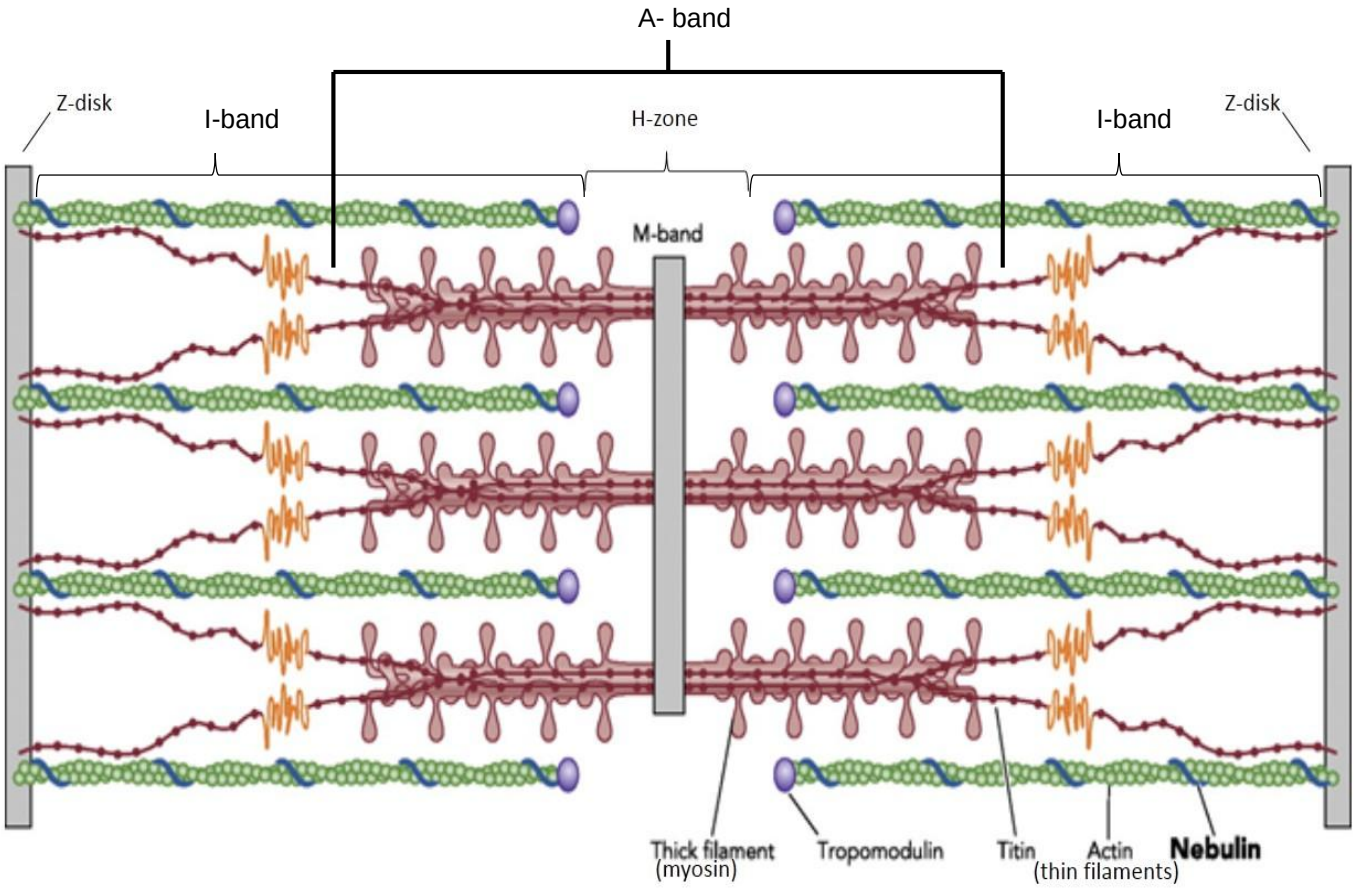


Figure 1.1 Sarcomere structure: Molecular representative of sarcomeres that make up the skeletal muscle. [Adapted from http://physiologyonline.physiology.org/content/25/5/304/F1.large.jpg]

1.1.1 Extracellular matrix

Within skeletal muscle, the extracellular matrix (ECM) also plays a crucial role and can be subdivided into three sections, namely; the endomysial (around the muscle fibre), perimysial (around bundles of muscle fibres) and epimysial (around the whole muscle) connective tissue (8) (see Figure 1.2 for schematic).

The ECM is associated with providing supporting scaffolding for the surrounding cells and tissues, leading to cell aggregation and also aiding cell migration (9). The ECM is a dynamic entity which is continuously modified, degraded and reassembled during states of development, disease and homeostasis (9–11).

The muscle ECM consists mostly of collagen, a major structural protein, that makes up 1-10% of muscle dry weight (12,13). Several types of collagen have been characterized within the skeletal muscle ECM, although fibrillar types I and III are the most predominant in adult ECM (14,15). Type

I collagen is suggested to be the main collagen type in the perimysium while type III collagen is evenly distributed between the endomysium and epimysium (15).

The ECM also contains an abundance of factors such as proteoglycans and glycosaminoglycans. Most of the proteoglycans in the muscle ECM are part of the small leucine-rich proteoglycans family (SLRPs) (16). This SLRP family has a core protein to which GAG (glycosaminoglycan) chains are attached. GAG chains consist of long linear carbohydrate polymers and examples of SLRP family members are decorin, biglycan, fibromodulin and lumican (17). Proteoglycans, more particularly, the negatively charged GAG chains, have the ability to regulate the bioavailability of some of the growth factors in the ECM surrounding the muscle (18,19). The ECM also contains enzymes like matrix metalloproteinases (MMP) that are able to cleave GAG chains, resulting in the release of growth factors such as fibroblast growth factor (FGF)(20) and transforming growth factor-β1 (TGF-β1)(21), to name but few. This enables them to be “free” and bind to their respective receptors to initiate signalling cascades (20–22).

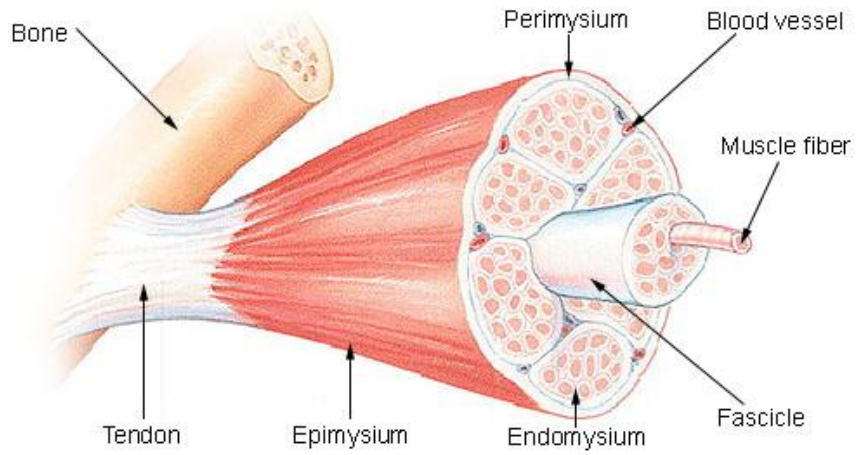


Figure 1.2 Basic structure of the skeletal muscle bundles: illustrating the bundle configuration of skeletal muscle and the role of the endomysium, perimysium and epimysium to support the structure of the muscle. [Adapted from http://training.seer.cancer.gov/anatomy]

1.1.2 Contracting muscle

Muscle contraction is initiated with an electrical signal leading to the release of acetylcholine (ACh) from the motorneurons onto the muscle fibres. The binding of the ACh changes the membrane permeability, resulting in an action potential that is conducted over the surface of the muscle membrane. The electrical signal is then relayed within the muscle, travelling to the sarcoplasmic reticulum (SR) via the T-tubules. This signal leads to the release of Ca^{2+} from the lateral sacs of the sarcoplasmic reticulum. The increased Ca^{2+} is crucial for muscle contraction as it enables cross-bridge cycling, by binding to troponin and displacing the tropomyosin on the actin, allowing

actin binding to myosin. During concentric contraction actin-myosin cross-bridge cycling results in sarcomere shortening leading to whole muscle shortening. (1)

1.1.3 Eccentric exercise induced skeletal muscle damage

Eccentric exercise, whereby the muscle actively produces force during lengthening, has been shown to produce a certain amount of muscle damage (23). It has been suggested that the exercise induced muscle damage is initiated by mechanical factors, which include: number of contractions, force, specific force and the velocity of the contraction. Greater muscle damage was found as the number of eccentric movements increased (24). Lieber and Friden (25) found that muscle strain during the lengthening contraction has a larger effect on exercise induced muscle damage than high forces. Furthermore, the lengthening velocity was concluded to be an important factor in increased muscle damage. These studies supported a mechanical initiation of exercise induced muscle damage.

When looking more closely at muscle mechanism during eccentric contraction, it has been shown that higher levels of force are produced while fewer motor units are activated, leading to the increased tension within the sarcomeres (26). This may lead to the disruption of myofilaments within the stretched sarcomeres. The augmented tension affects especially the Z-disk area (39), leading to disruption of the sarcomeres particularly at the Z-disk regions (23,28–30). The detrimental effects on the Z-disks lead to damaged cytoskeletal proteins that are normally vital in the maintenance of sarcomere structure (31). Furthermore, when the eccentric contraction is repetitive with increased intensity, the tension is augmented and may be expanded to the adjacent sarcomeres, resulting in a decrease in the amount of intact sarcomeres (32).

There are many factors contributing to the muscle damage, including the activation of ion channels leading to the increase of intracellular calcium levels resulting in the activation of calpains (33). These calcium-activated proteases cleave important structural proteins including titin, desmin, troponin and tropomyosin (33).The damaged muscle also initiates an inflammatory process, with increases in macrophages and neutrophils within the muscle (34).

Nonetheless, the mechanical stress placed on the muscle during the lengthening contraction seems to be the initial incident leading to subsequent events which end up with the damaging of intracellular proteins, decrease in force and increased muscle soreness, which peaks at 24-72 hours after exercise (35,36). The muscle soreness, more commonly known as DOMS (delayed onset of muscle soreness), is not correlated with the magnitude of the muscle damage induced by the exercise (37). Release of various muscle proteins into the circulation also do not correlate directly with the magnitude of damage. However, both these variables may be used as an indirect indication that damage has occurred, irrespectively of the amount.

1.1.4 Indirect muscle damage indicator - creatine kinase

Creatine kinase (CK) is an enzyme with a molecular weight of approximately 82 kDa and is found in the cytoplasm and mitochondria of cells with a high energy demand such as skeletal muscle. The muscle specific creatine kinase consists of two identical sub-units, also known as the muscle type subunits (M) forming a primary MM enzyme. The mitochondrial creatine kinase found in skeletal muscle is known as the Mt-CK (38). The function of creatine kinase relates to the reversible phosphorylation of creatine (39).

Once creatine is phosphorylated by creatine kinase, the phosphocreatine then transfers a high energy phosphate group to ADP to form cellular energy unit ATP (40,41).

$$Phosphocreatine + MgADP \leftrightarrow MgATP + creatine$$

This emphasizes the importance of creatine kinase in producing energy and forming the core of the phosphocreatine energy system.

In the case of eccentric exercise, skeletal muscle is exposed to unaccustomed muscle action which leads to variable degree of mechanical muscle damage (42). The accompanying metabolic disturbance is the possible cause of the released cellular components, occurring in a sequence: First a reduction of ATP and the reduced uptake of extracellular calcium into the sarcoplasmic reticulum due to the dysfunction of associated SR membrane ATPase; this is followed by the increase and activation of intracellular proteolytic enzymes leading to the increased degradation of muscle proteins, resulting in increased leakage of cellular components (43,44) (see Figure 1.3). In healthy individuals exposed to isolated mild to moderate damage it was shown that the clearing of circulating muscle components occurs within 7-9 days (45,46).

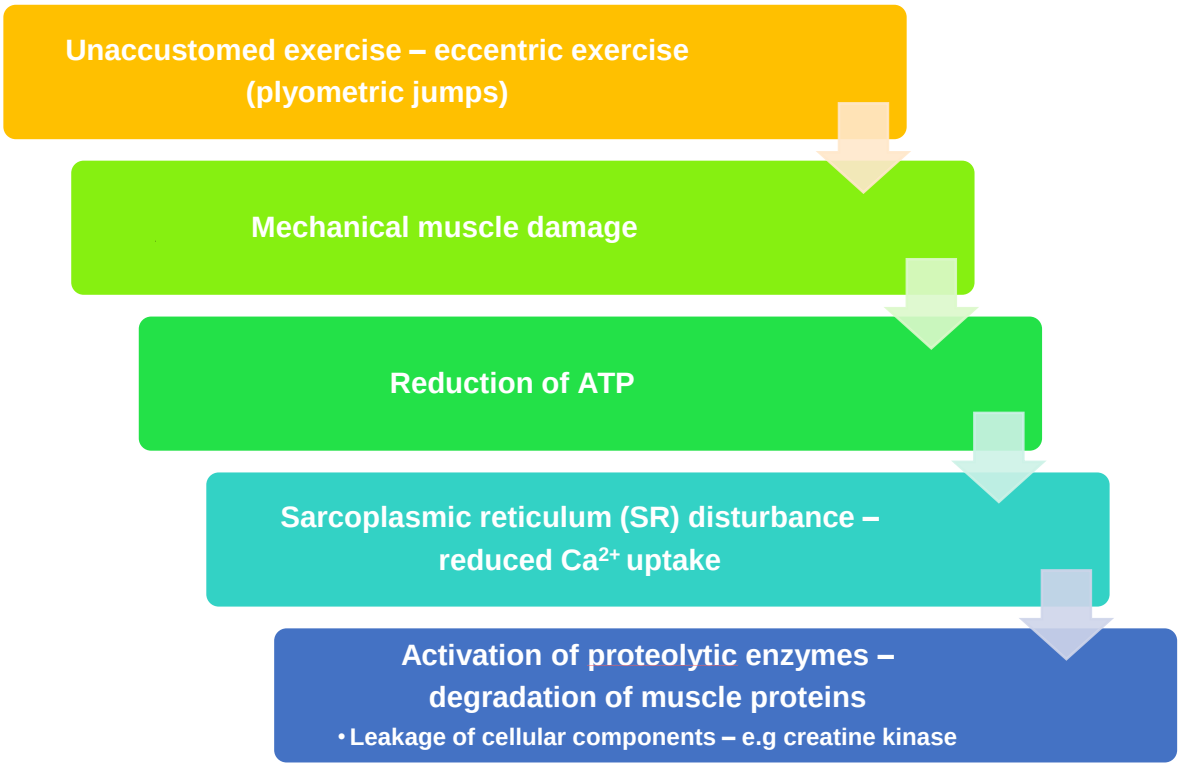


Figure 1.3 The sequence of events leading to the increase in serum creatine kinase observed after exercise induced muscle damage

The same exercise protocol may lead to different levels of creatine kinase in the blood, even in a group of subjects with similar age, gender, and training status. In many cases, the reason for this is unknown, although in some cases it is possibly an effect of muscle pathology like myopathy or and cardiomyopathies (47–50).

The baseline serum CK levels in the general population varies from between 35 - 176 U/L (51), but in the examples of individuals suffering from rhabdomyolysis, CK can be between 10,000 – 20, 000 U/L and can achieve levels as high as 3×10^6 U/L (52). This abnormally high level of CK indicates a strong disturbance of striated muscle, leading to this increased efflux of intracellular muscle components. As a guideline, it is suggested that serum CK levels higher than 5,000 U/L occurring in the absence of myocardial or brain infarction, physical trauma or disease, serves as an indication of serious muscle disturbance (44).

1.1.5 Connective tissue damage associated with eccentric muscle action

Aside from the commonly discussed muscle damage associated with eccentric exercise, there is also the aspect of connective tissue damage accompanying this mode of exercise (53). A study using an animal model showed an increase in collagen content when rats were exposed to 4 weeks of lengthening contractions (54). This may be part of the adaptation following eccentric

exercise, as it was demonstrated that increased connective tissue is linked with a decreased injury response to subsequent lengthening contractions (55).

The direct investigation of connective tissue damage in human subjects is limited; however indirect methods are used to evaluate the damage. One of the indirect markers that has been used is hydroxyproline, and a study investigating Navy Seals during an intense physical training program showed elevated levels of hydroxyproline associated with activity induced connective tissue damage (56). In contrast, concentric exercise did not result in changes in serum hydroxyproline (57). Aside from the hydroxylated-amino acids, elevated pyridinoline levels in the urine after eccentric exercise, are also associated with collagen breakdown (53).

A study by Brown et al. (53) investigated the differences in response to maximal force production and different indirect markers of damage in response to a bout of concentric exercise followed by a bout of eccentric exercise. The results showed a significant increase in the CK and lactate dehydrogenase isoenzyme (LDH-1) levels after the eccentric exercise. There were no changes in serum hydroxyproline, but increased levels of collagen were observed on days 1 and 9 after exercise. The maximal voluntary isometric contraction force (MVC) of the subjects was also affected and testing showed decreased MVC immediately after the eccentric contractions (42).

1.1.6 Force production following exercise induced muscle damage

Eccentric contractions are responsible for a decrease in force production due to the increased muscle damage associated with lengthening contraction (58,59). The lengthening contractions overstretch the sarcomeres leading to reduced contractile protein cross bridge interaction (60) as well as excitation-contraction coupling (61), subsequently resulting in decreased strength. Maximal voluntary contraction (MVC) during isometric contractions is commonly used to assess strength after eccentric exercise and is considered a good tool for indication of damage (62). During testing, isometric contractions are held for 2-5 seconds at a set angle. This method has been used for testing force production in elbow flexors and knee flexors after eccentric exercise (63), however it seems that the elbow flexors (63) are more affected by damage compared to the lower limbs (64). This is evident in the 50-60% (63,65,66) strength reduction in elbow flexors compared to around 35% (67–69) in knee flexors following exercise. In more intense eccentric protocols 50-70% reduction in strength were reported, with recovery time possibly spanning over several weeks (70).

1.2 Skeletal muscle responses: regeneration and adaptation

Skeletal muscle responses to damage include both regeneration and adaptation. Repair is a complex interplay of molecular factors and successful repair is dependent on the synchronization of inflammation and regeneration (71,72). Muscle repair consists of two phases: degeneration and regeneration. These two phases are dependent on each other. The sequence for muscle repair remains the same, but can be affected by the cause of the damage, kinetics of each phase, magnitude of the damage and the damage model used (71,73–76). When damage is induced by exercise, exercise-induced adaptations may also be stimulated to reduce the likelihood of damage if the same insult was experienced again in the future (77–79). A lot of attention has been paid to the mechanisms of repair following eccentric exercise (80,81), but less attention has been paid to the stimuli for adaptation. This is complicated by the fact that some molecular responses are most likely common to both processes.

The most important aspects of muscle repair are the activation, proliferation and differentiation of cells aiding in the rebuilding process. This introduces the role of satellite cells and their myogenic regulatory factors in muscle regeneration.

1.2.1 Satellite cells and myogenic regulatory factors in regeneration

Satellite cells are a small population of muscle precursor cells discovered by Mauro in 1961 (82) and they give skeletal muscle the ability to adapt and regenerate after damage. In the absence of muscle damage, these satellite cells remain quiescent in their niche between the basal lamina and sarcolemma (83). In the instance of myotrauma, satellite cells are activated and proliferate increasing their numbers and start expressing myogenic markers. These activated satellite cells, also known as myoblasts, can then fuse with existing muscle fibres or form new myofibres, subsequently aiding in the muscle regeneration process (84,85).

The satellite cells in intact muscle are mainly in a state of mitotic quiescence (G_0). These quiescent cells are commonly characterized by being $Pax7^+/MyoD^-/Myogenin^-$ (86). In the presence of damage, these satellite cells are activated and start to proliferate exiting their state of quiescence. The activation of satellite cells is controlled by several factors affecting the satellite cell niche and signalling pathways. Interestingly, the activation of satellite cells is not only limited to the site of the myofibre injury, as satellite cells from different areas of the myofibre can be activated and induced to migrate to the site of injury (87). This occurrence is facilitated by the regulation of the anti-adhesive molecule, sialomucin CD34 (88). *In vitro* evidence suggest that regulation of Eph receptors and ephrin ligands in satellite cells also aid in directing satellite cell migration (89)

MyoD is part of the group of the bHLH (basic helix-loop-helix) proteins, which is associated with muscle differentiation although its upregulation already occurs during proliferation (90). The MyoD gene is considered as an example of a master control gene in the differentiation process (91).

MyoD has been shown to be capable of fully activating the muscle differentiation program in both *in vitro* and *in vivo* models (92). MyoD was found to be responsible for promoting gene expression of proteins like desmin and myosin heavy chain, even in non-muscle cells (92). It has also been found that MyoD has a binding site in the upstream promoter region of the myostatin gene. Since myostatin is a muscle-specific anti-growth factor, this suggests a link or regulation system existing between MyoD and myostatin that could, together, have powerful regulatory effect on satellite cell differentiation (80, 92). This could be independent of the effects on muscle anabolism or atrophy (see 1.2.2).

The other members in this group include Myf5, MRF4 (myogenic regulator factor) and Myogenin. During the formation of muscle, Myf5 and MyoD play an important role in the establishment and maintenance of the muscle progenitor cells, while MRF4 plays several roles in myogenesis. Lastly, Myogenin is important for terminal differentiation. These bHLH proteins consists of four conserved domains: 1) a section known as TAD (transcriptional activation domain), making up the amino terminal region 2) cysteine/histidine rich region 3) basic-helix-loop-helix region in the middle and 4) α-helix domain at the carboxy terminal (93,94). The TAD domain is known to be a potent transcriptional activator (95) while the cysteine/histidine (C/H) domain along with the α-helix domain are involved in chromatin remodelling, allowing previously repressed muscle specific genes to be expressed (94). The bHLH region within these transcriptional factors is important for the binding to the E-box consensus sequence, located in the regulation control regions of several muscle specific genes (96–98).

During the process of muscle regeneration, both MyoD and Myogenin play important roles in the promotion of muscle specific genes (99–102) leading to differentiation in activated muscle satellite cells (see Figure 1.4). These two factors are able to bind to the E-box sequence (CANNTG) in the promoter region of muscle specific genes. Studies investigating cultured MyoD -/- myoblasts revealed a reduction in myogenic specific gene expression leading to delayed differentiation e.g. lower levels of myosin heavy chain, myogenin, MRF4 and acetylcholine receptor- δ (103–105).

Myogenin is also one of the basic-helix-loop-helix (bHLH) transcription factor which forms part of the myogenic regulatory factor (MRF) family (106–111). This MRF family members have the ability to regulate expression of each other and other muscle specific muscle proteins (91,112,113).

Other pathways have also been implicated in the activation of satellite cells (114–116) and MRFs including several factors e.g. HGF (117), FGF (118), IGF (119,120) and nitric oxide (121).

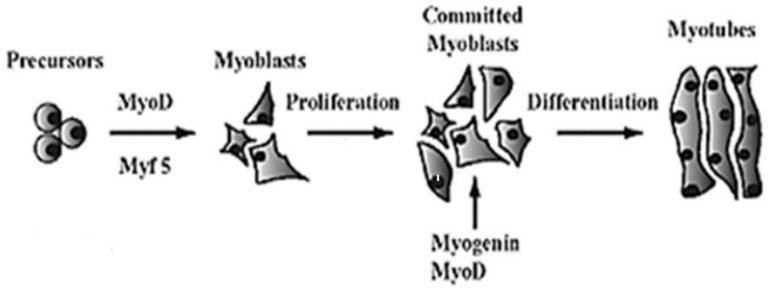


Figure 1.4 Myogenic pathway: illustrating the progression of satellite cells to myotubes and illustrating the stages at which myogenic transcription factors are involved. [Adapted from Langley et al. (81)]

1.2.2 Hypertrophy and Atrophy

The increase in skeletal muscle mass in response to a weight bearing stimulus is associated with the increase in signalling through the PI3K/Akt pathway, leading to the downstream activation of factors increasing protein synthesis (122,123). Insulin-like growth factor-1 (IGF-1), is one of the main contributors to hypertrophy and one of the agents able to induce the activation of the PI3K/Akt pathway (122,123). In response to muscle overload, IGF-1 is locally produced and initiates the Akt pathway leading to the resulting muscle hypertrophy. The effect of IGF-1 was illustrated when mice were genetically modified to overexpress IGF-1, showing a dramatic increase in myofiber hypertrophy (124). Studies in Drosophila aided in constructing the map for the Akt pathway and revealed that IRS-1 (125), P13K (126), mTOR (127) and p70S6K are important for skeletal mass and without any of these factors there is a decrease in cell size. Regulation of cell size is therefore controlled by signalling pathway responses to mechanical loading or growth factors, or both.

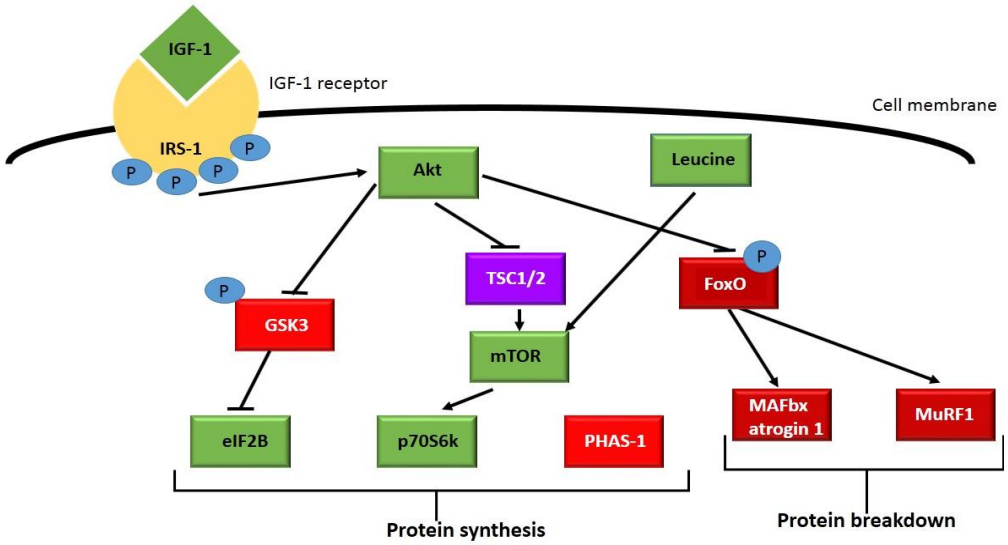


Figure 1.5 Schematic of the signalling involved in hypertrophy and atrophy within skeletal muscle

In the instance of IGF-1, mTOR is activated downstream of Akt, however, it has been found that the amino acid Leucine directly increases mTOR signalling and subsequently augments protein synthesis (128).

It seems that mTOR serves as a master regulator of the pathway and integrates a variety of signals, affecting protein synthesis. Proof of its central role has been provided *in vitro*. The inhibition of mTOR, by a pharmacological agent in cell culture, inhibited the activation of p70S6K which attenuated the anabolic outcome of Akt or IGF-1 (123,129,130)(see Figure 1.5).

Studies have provided evidence that exercise induced muscle damage enhances IGF-1 production leading to an anabolic response (either repair or hypertrophic) associated with the exercise. A study by Bamman et al. (131) revealed that although concentric exercise does not lead to a significant change in IGF-1 or IGF-binding protein-4 (IGFBP4) mRNA levels, the opposite is true for eccentric exercise. However, IGF-1 mRNA was significantly increased in response to eccentric exercise while IGFBP4 was significantly decreased, suggesting the link between muscle damage (prominent with eccentric exercise) and IGF-1 response (131). Furthermore, all three isoforms of IGF-1 (IGF-1Ea, IGF-1Eb and IGF-1Ec, also known as Mechano Growth Factor (MGF)) were investigated by McKay et al. (132) in an *in vivo* setting. The study used eight healthy male subjects to perform 300 knee extensors lengthening contractions. Analysis revealed that MGF mRNA were significantly increased at 24 hours after the exercise protocol while the other two isoforms exhibited no increase until 72 hours post exercise (132). The timing of MGF expression post exercise, suggests that MGF plays an important role in the repair process in response to exercise induced muscle damage.

The inhibition of glycogen synthase kinase 3-beta (GSK3β) by Akt is an additional method in which hypertrophy is increased, as the expression of inactive GSK-3β resulted in skeletal myotubes hypertrophy (123,133). Interestingly, by decreasing the activity of GSK3β, there is higher activity of translational initiation factor eIF2B (134) which suggests that protein synthesis can be upregulated without the activation of mTOR (see Figure 1.5). This was supported in models using Wnt1, a known inhibitor of GSK3β (135). Members of the Wnt family are also known to play a role in satellite cell regulation, possibly providing for integration between muscle fibre hypertrophy and satellite cell activation/fusion (136–138).

Aside from the ability of Akt to initiate the anabolic signalling leading to protein synthesis, it also decreases signalling through the atrophy pathways responsible for protein breakdown. *In vitro* studies using dexamethasone to induce atrophy have shown that upregulated MAFbx and MuRF1 could be antagonized by IGF-1 treatment, also initiating the P13K/Akt pathway (139–141). Muscle size is dependent on the balance between pro- and anti-anabolic signals induced by pro-growth or anti-growth factors, respectively. The interconnection is complex. For example, the downregulation

of MuRF 1 and MAFbx is dependent on the effect of Akt on FoxO transcription factors which regulate expression of these ubiquitin ligases. When FoxO is phosphorylated by Akt it is excluded from the nucleus (140).

1.3 Significant roles of Myostatin in skeletal muscle

1.3.1 Myostatin/ Growth differentiation factor-8 (GDF-8)

Myostatin, a well-known negative regulator of muscle mass, was first reported in 1997 in research by McPherron et al. (142) in a study in which the myostatin null mice, resulted in 2-3 times larger muscles compared to the wild type mice. Following the original discovery of myostatin, several papers were published associating myostatin gene mutations with double-muscled cattle, (143–146) confirming the powerful effect of myostatin on muscle mass regulation. A prominent study described the phenotype of a boy, who at birth, was normal apart from the clear increase in muscle mass and 7 months after birth the child had clearly defined, big thigh and calves muscles. At the age of 4 years and 6 months, the child had the ability to hold two 3 kg dumbbells with his arms horizontally extended. Genome analysis of the boy highlighted the presence of a mutation in the myostatin gene that affected the splicing of the mRNA gene and hence the insertion of a premature termination codon, thus negatively impacting on the production of functional myostatin (147).

Conversely, *in vitro* treatment of C2C12 and L6 skeletal muscle cells with the atrophy-inducing agent dexamethasone (a cortisol mimetic) showed an increase in both myostatin mRNA and protein levels (148). The study suggested that atrophy associated with glucocorticoids may be due to this upregulation of myostatin, as a negative muscle mass regulator (148).

The atrophy caused by myostatin in myotubes is as result of the inhibition of Akt by myostatin-Smad signalling (149). As mentioned earlier, the inhibition of Akt leads to reduced phosphorylation of FoxO (139,140), thereby permitting the translocation of FoxO to the nucleus resulting in the upregulation of E3 ligases MuRF1 and MAFbx, which are associated with atrophy (139,140). Furthermore, myostatin also affects satellite cells during activation (150) and proliferation (151,152). Cell studies have revealed that myostatin halts the progression of the cell cycle at G1 and G2 phases, due to p21 upregulation by myostatin (see Figure 1.6). Myostatin also decreases the concentration of Cdk2, which along with increased p21, leads to hypophosphorylation of retinoblastoma protein (Rb). This stops the progression into the S-phase (151). In addition, the myostatin-Smad signalling pathway inhibit MyoD and Myogenin in a Smad3 dependant manner, leading to altered differentiation of myoblasts (81).

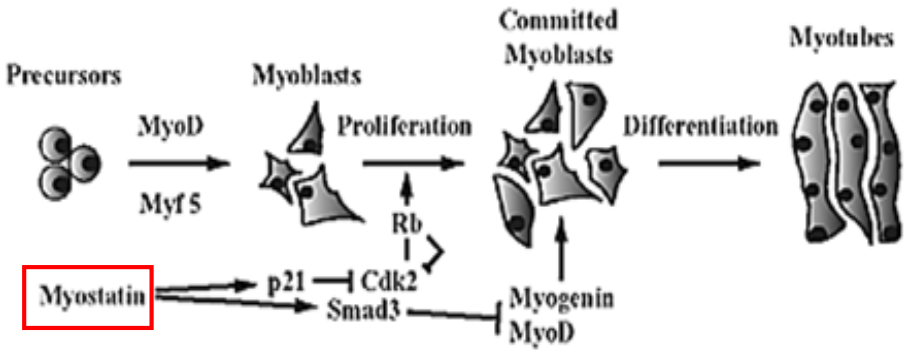


Figure 1.6 Myogenic progression influenced by myostatin - Illustrating the influence of myostatin on myoblast proliferation and differentiation [Adapted from Langley et al.(81)]

These findings initiated interest in the possibility that silencing or inhibiting myostatin may reverse or aid individuals suffering from loss of muscle due to illness or genetic mutations.

1.3.2 Myostatin in human models of atrophy

The increase in myostatin is regularly associated with illness-related muscle wasting or cachexia observed in people suffering from acute immunodeficiency syndrome (AIDS)(153), heart failure (154–156) and kidney disease (157). The decrease in muscle mass has a major effect on the quality of life and has been linked to increased mortality (158).

In individuals (males) suffering from AIDS, the active mature myostatin protein is increased within the serum and skeletal muscle, when compared to healthy males (153). Patients with stage 5 chronic kidney disease also show significantly higher levels of myostatin expression, while the expression of IGF-1 was attenuated, when compared to the control. These factors are two of the most powerful regulators of muscle mass and this unfavourable balance towards myostatin expression might be the main cause associated with muscle wasting in patients with kidney disease (159).

Not only disease, but disuse of muscle (e.g. bed rest or limb immobilization) also leads to atrophy within the skeletal muscle and it has been shown that quantifiable muscle atrophy is evident after only 5 days of muscle disuse (160). Furthermore, the same study revealed that muscle myostatin mRNA expression doubled in groups exposed to the 5 day- and 14-day leg immobilization and that myostatin precursor protein decreased only after 14 days (160).

The negative effects of myostatin associated with illness and the potential advantages that may be gained by athletes if myostatin can be regulated or inhibited has been a massive motivational factor for research into myostatin. At the moment the number of human studies is limited since animal models are easier to manipulate and in some instances naturally occurring genetic mutations (143,161) are evident.

1.3.3 Human exercise studies investigating myostatin

Investigation into myostatin in humans, show the involvement of myostatin in adaptation to both endurance and resistance exercise.

When myostatin mRNA expression is analysed in physically active men and woman after a single bout of 30 min running, it was found that expression was decreased up to 4-fold at 8 h and 12 h post exercise (162). Similar decreases were observed in the *soleus* and *vastus lateralis* of trained men, measured in biopsies 4 h after 45 min running bout at 75% of their $VO_{2\ max.}$ (33). Furthermore, the study reported no changes at 24 h after exercise (163).These results suggest a consistent response observed in both chronic and acute endurance training.

In the instance of resistance exercise, myostatin mRNA expression is decreased after resistance training in males and females, irrespective of age (164). The expression still seems to be decreased as late as 48-72 h after the final bout of near maximal resistance training (164). Similar decreases were observed after an acute bout of exercise, resulting in a 44% decrease at mRNA level for biopsies taken 24 h after exercise in young and old individuals (males and females)(165).

In contrast, a study by Willoughby (166), investigated myostatin at mRNA and protein level in muscle and also included serum myostatin concentration. This study reported an increase in total myostatin measured 15 min after a 6 or 12 week resistance training program. The training protocol consisted of training 3/week using three sets of six to eight repetitions at 85-90% 1-RM on lower-body exercises, whereas CON performed no resistance training (166). In another study, myostatin mRNA measured 4 h after a single bout of leg extensions performed by endurance-or resistance trained subjects, were found to be unchanged (167). Expression information reported by Hulmi et al. (168), showed mRNA levels were not affected 1 h after exercise, but were indeed significantly decreased 48 h after the final bout of a 21-week training regime. The results from the latter study possibly explain the differences in myostatin expression reported at various time points after exercise. It would seem that expression is only affected at a later time point, maybe only as late as 48 h after exercise.

When investigating myostatin at the protein level (analysis using Western blotting), it has been reported that plasma myostatin concentrations were decreased by 20% after exercising twice a day for 10 weeks (169). Similar results were found in a study by Saremi et al. (170), who reported a 10% decrease in plasma myostatin (analysis was performed using ELISA) after 12 weeks resistance training while supplementing subjects with creatine. Again, controversial results pertaining to serum myostatin were reported with Kim et al. (171), who reported no changes at protein level after 16 weeks of knee extensor resistance training. The study also revealed a high level of variance in serum myostatin levels between untrained individuals (171).

In summary, these studies provide evidence that is somewhat contradictory and emphasizes the point that exercise mode, duration of study, time point of sample harvesting and training status all have effects on protein expression. Furthermore, analysis of myostatin protein expression is often incomplete: somewhat lacking in detailed analysis providing complete information concerning the processing and activity status of the myostatin and the activation of targeted signalling pathways.

1.3.4 Myostatin expression and myostatin propeptide

Physiologically, the expression of myostatin is largely influenced by the FoxO1 transcription factors (172). It was found in C2C12 cells that FoxO1 induced expressed of myostatin via its binding site in the promoter region of the myostatin gene (172). Furthermore, results from the same study revealed the involvement of Smads in the expression of myostatin. Over-expression of the Smad transcription factors led to increased myostatin promoter activity (172).

Myostatin gene expression is followed by translation into a precursor protein consisting of 375 amino acids (aa), including the signal sequence (23 aa), N-terminal propeptide domain (243 aa) and a C-terminal domain (109 aa). The precursor protein is cleaved at two sites to produce an active form of myostatin. This occurs in several steps. The furin family of enzymes is responsible for the first cleavage, removing the 23 aa signal peptide (173)(see Figure 1.7 Step 1). The next cleavage by bone morphogenetic protein-1/tolloid (BMP-1/TLD) occurs at the Arg-Ser-Arg-Arg (RSRR, aa 263-266) site separating the N-terminal domain (26-27 kDa) from the C-terminal domain (12-13 kDa)(173)(Step 2). Two C-terminal domains form a disulfide-linked dimer (26 kDa). Two N-terminal domains/myostatin propeptide binds non-covalently to the dimer, forming an inactive (latent) complex (174,175)(Step 3). The BMP-1 enzyme family of metalloproteinases has been found to be responsible for cleaving the inactive complex between Arg-75 and Asp-76, enabling the active (also called the mature) myostatin to then bind the designated receptor (see Figure 1.7, Step 4)(176). – *All myostatin protein sizes is in accordance with information from UNIPROT online database.*

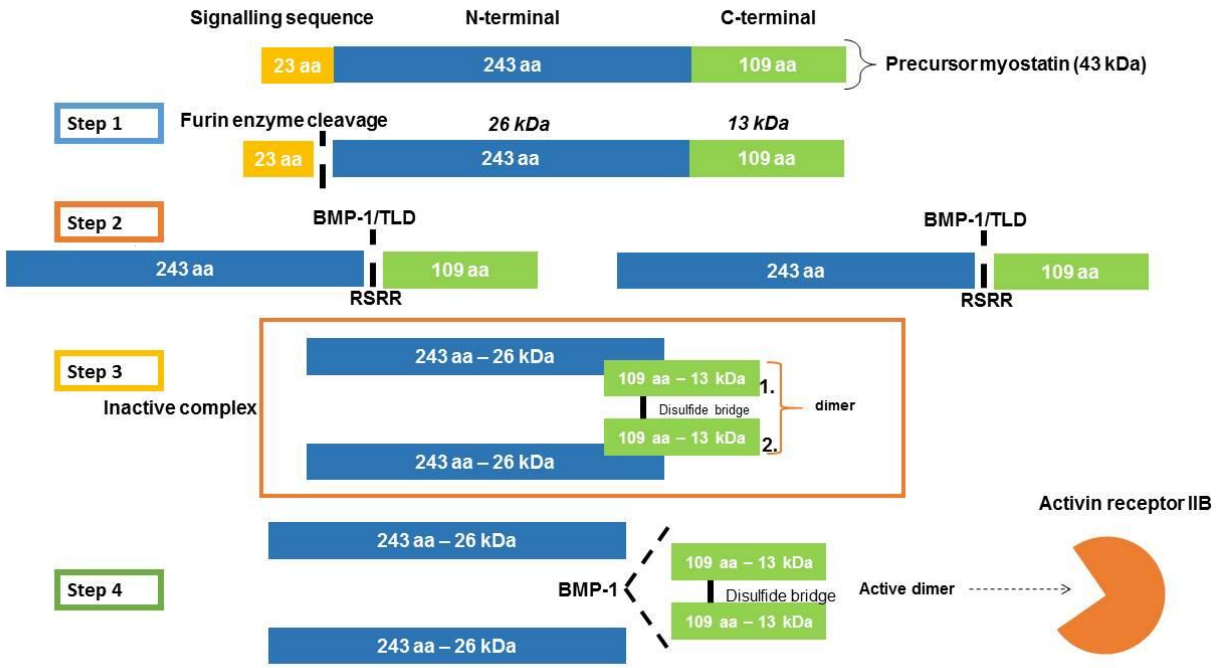


Figure 1.7 Myostatin processing and secretion - Illustrates the processing of myostatin after expression and the enzymes involved in releasing the active myostatin that binds to the designated receptor.

The myostatin propeptide has been shown to inhibit the biological activity of myostatin *in vitro* (see Figure 1.9)(174). This occurrence was observed *in vivo* as well, with results showing that a high percentage of mature myostatin in normal mouse and human serum is bound to the propeptide (177). Furthermore, a significant increase in muscle mass was seen in normal wild-type mice and mice suffering from Duchenne muscular dystrophy when gene delivery of myostatin propeptide was administered (178).

1.3.5 Myostatin designated receptor

It is well known that the members of the TGF-β superfamily initiate signalling by binding to serine/threonine kinase receptors (175). The activin IIb receptor (ActRIIb) is a serine/threonine kinase receptor and was identified as the primary receptor for myostatin. Lee and McPherron showed that expression of a dominant negative form (lacking the kinase domain of the receptor) of the ActRIIb led to an increase in muscle mass of 125% when compared to control/non-transgenic animals (175). Myostatin binding is specific and saturable (175) and leads to activation of members of the Smad signalling pathway.

1.3.6 Signalling - Smad dependent

The human genome encodes for a total of eight Smad proteins (179), with five of them (Smad1, Smad2, Smad3, Smad5 and Smad8) acting as substrate proteins for the TGF-β family of receptors. These are commonly referred to as receptor-Smads (R-Smads). Smad4, also known as the co-Smad, is able to serve as a common partner for all R-Smads (179). Smad6, 7 are known as the inhibitors of the pathway by interfering with Smad-receptor or Smad-Smad interactions (179).

The proteins themselves consist of approximately 500 amino acids and have two globular domains separated by a linker region (180). The C-terminal (MH2, homology domain) is conserved in all Smad proteins (see Figure 1.8) while the N-terminal (MH1, homology domain) is conserved in all Smads proteins excluding Smad6 and Smad7 (see Figure 1.8). The MH1 domain seems to be responsible for DNA-binding and is stabilized by a tightly bound zinc atom. The linker region serves as a binding target for an array of proteins including Smurf (Smad ubiquitination-related factor), phosphorylation sites for several protein kinase and ubiquitinase ligases. In Smad4, the linker site is the binding region for proteins, nuclear export signal (NES), affecting its localization (180) (see Figure 1.8).

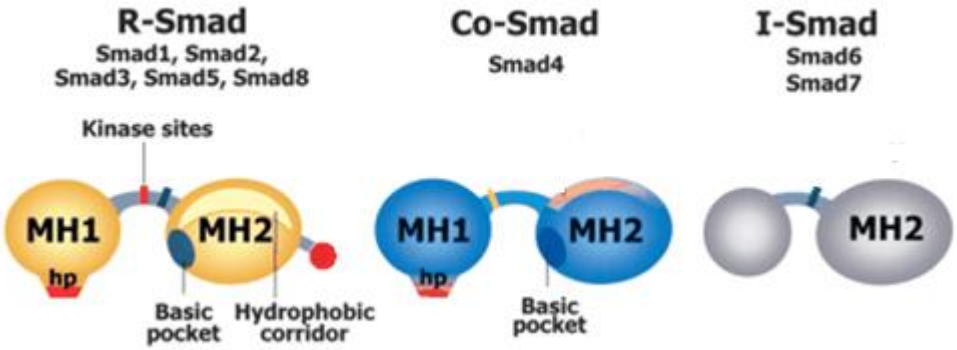


Figure 1.8 Structures of Smad proteins involved in TGF-β signalling [Adapted from Massagué et al. (165)]

As mentioned above, in the case of active myostatin, the dimer binds with high affinity to the Activin receptor IIB (ActRIIb) (175,181). The ActRIIb receptor consists of a short extracellular domain which binds the ligand and then a large intracellular portion possessing the serine/threonine kinase domain (182,183). Signalling is initiated when the ligand, like myostatin, binds ActRIIb receptor which in turn forms a complex with the Anaplastic lymphoma kinase (ALK) receptor 4 or 5 (subtypes of type I receptor). This complex phosphorylates, via the serine/threonine kinase domain (184). The serine-threonine kinase subsequently phosphorylates Smad2/3 proteins, also known as receptor-regulated Smads. The phosphorylated Smad2/3 is then able to form a complex with Smad4 to translocate into the nucleus leading to the repression or upregulation of specific genes (See Figure 1.9)(185).

1.3.7 Inhibitory Smad 7

Interestingly, myostatin is subject to autoregulation. This occurs via the expression of Smad7, a downstream gene target of myostatin/Smad2/3 (186). It was also discovered that overexpression of Smad7 led to inhibition of activity at the myostatin promoter site (186). Furthermore, research by Nakao et al. (187) found that Smad7 is one of the inhibitory Smad (I-Smad) proteins and is capable of reducing signalling through Smad2/3 by interfering with the phosphorylation of these proteins (see Figure 1.9)(187). The Smad7 protein also interacts with MyoD and enhances its transcriptional activity (188). Taken together, Smad7 plays a major role in differentiation in skeletal muscle cells (188).

1.3.8 Regulation of myostatin

Myostatin is regulated by a variety of proteins/factors at protein level, for example proteins; interfere with the binding of myostatin to the receptor or via inhibition of the Smad signalling pathway. The main regulatory factors involved include myostatin propeptide (174), follistatin (189), Smad7 (186,187), Tcap (telothonin)(190) and more recently decorin (191).

1.3.8.1 Follistatin

Follistatin (FST) is a multi-domain factor able to bind and regulate members of the TGF-β superfamily, particularly Activin A and myostatin (see Figure 1.9)(189). The affinity for Activin A and myostatin differs, with Kd values of 1.7 nM and 12.3 nM respectively (192). Follistatin type proteins are subdivided into follistatin and follistatin-like 3 (FSTL-3). The two proteins differ from each other in their molecular structure, binding characteristics and their affinities for members of the TGF-β family.

The follistatin protein contains an N-terminal domain and three FST domains (FS Domains 1-3), with a heparin-binding site in FS Domain-1 (189,193). The heparin binding site is involved in regulation of myostatin via degradation (194). This occurs as follistatin binds myostatin, which increases the affinity for cell surface localized heparin (194). The binding of follistatin with heparin facilitates the endocytosis of follistatin-bound ligands (189,193).

The crystal structures of FST proteins in complex with myostatin or closely related activin A, showed that follistatin inhibits the action of these ligands by blocking all four receptor-binding sites (194–198). The analysis shows that the FS Domain-1 and FS Domain-2 are responsible for covering the type II receptor-binding site, while the type I receptor binding site is occupied by the

N-domain. There are also interactions between follistatin proteins with the N-domain of one molecule interacting with the FS Domain-3 of another (192,194,199,200).

The physiological importance of FST was emphasized with studies in which follistatin was overexpressed and showed that myostatin effects were decreased leading to a radical increase in muscle mass (175,201). In a study by Gilson et al. (202), transgenic mice were modified to over express follistatin. The increase in follistatin protein concentration within the muscle induced skeletal muscle hypertrophy, including the activation of satellite cells. The hypertrophy induced by follistatin was characterized by the increase in DNA content which reflects the number of myonuclei. This study also supported the ability of follistatin to inhibit the effects of activin and myostatin, leading to increased hypertrophy. In the same study, γ-irradiation was used to destroy satellite cells' ability to proliferate. The muscles were then treated with follistatin by gene transfer and the results showed that follistatin is able to induce hypertrophy through protein synthesis. This shows that follistatin can affect muscle mass in a satellite cell independent manner (202).

Furthermore, a study by Lee (203) investigated the influences of other ligands in the absence of myostatin. The study used Mstn-/- mice which carried a follistatin transgene. The combination of follistatin transgene with myostatin null mice resulted in a quadrupling in muscle mass. These mice were double the size of the myostatin-null mice. The muscle mass increase included a 73% increase in fibre number and a 117% increase in fibre cross sectional area, compared to the wild type mice. These results emphasize the ability of follistatin to regulate other ligands involved in muscle mass regulation. (203)

The regulation of follistatin expression is not completely clear. However, a link between IGF-1 and follistatin expression has been established in quail (204) and duck (205). In the quail, *in ovo* feeding with IGF-1 significantly altered the follistatin expression levels in the developing muscle tissue (204). While in duck embryos, the results showed a similar expression pattern for follistatin, under the influence of IGF-1: *in ovo* IGF-1 administration also resulted in an increase in follistatin expression within developing skeletal muscle (205). It is therefore suggested that IGF-1 may be one of the factors leading to an increase in follistatin mRNA expression (205).

Studies investigating the response of follistatin mRNA levels to exercise, both concentric and eccentric, have reported no significant changes (206,207). In the most significant of these studies, young males were recruited (along with older participants, results not discussed here) and were subjected to six sets of 12-16 maximal eccentric repetitions of single-leg eccentric knee extension on an isokinetic dynamometer (206). Skeletal muscle biopsies were taken before the start of the study and then 24 h after the exercise protocol, followed by mRNA analysis revealing no change in follistatin expression in young men when subjected to eccentric exercise (206).

1.3.8.2 Decorin

Decorin is an important regulating member of the dynamic extracellular matrix and is part of the family of small proteoglycans. Decorin binds to several substances in the ECM and regulates collagen fibril formation and stabilization of the collagen network (208,209). Aside from the structural effects of decorin, it also plays a regulatory role (interference, regulation or stimulation) in a variety of pathways and with specific factors. One of the most prominent effects, fibrosis, may be due to the capability of decorin to sequester TGF-β1, thereby decreasing the fibrotic response (210,211).

In 2006, a study by Miura et al. (191) introduced another role for decorin showing its ability to bind myostatin (see Figure 1.9), another member of the TGF-β superfamily. The *in vitro* studies showed improved myoblast proliferation in the presence of immobilized decorin, relieving the negative effects of myostatin on myogenic cell proliferation (191).

In a study by Li et al. (212) the *in vitro* effect of decorin on the differentiation of myoblasts was investigated. The study went further and also looked at the *in vitro* and *in vivo* behaviour of myoblasts transfected with the decorin gene (212). Of interest to the current project, the over-expression of decorin was shown to upregulate follistatin, p21 and myogenic transcription factors while also down-regulating myostatin. All these effects, along with decorin's ability to sequester TGF-β1, may explain the enhanced myogenic differentiation and decreased fibrosis observed in the presence of increased decorin (213–217).

Further work was done by Kishioka et al. (218), in which the investigators generated a C2C12 model over-expressing decorin. The results showed that free decorin affected myogenic cells by enhancing their proliferation and differentiation through interfering with myostatin signalling. The results also indicated that the cells over-expressing decorin showed significantly larger myotubes at 96 hours. This was accredited to the fact that myostatin inhibition leads to extended proliferation (or delayed cell cycle exit) allowing for more myoblasts to ultimately fuse and differentiate. Furthermore, the study also found that over-expression of decorin does not affect myostatin expression, but decreases the ability of myostatin to elicit its physiological effects (218). To gain a better understanding of the multiple effects of decorin, it is useful to understand its effect in biological systems other than muscle.

In vitro, the expression of decorin has also been investigated in human keratinocytes (cHEK cells) and a link was revealed between decorin mRNA expression, pro-inflammatory and proliferative cytokines (219). The study revealed that treatment of cHEK cells with IL-1β and TNF-α inhibited expression of decorin (219). Furthermore, the addition of TGF-β1 resulted in an 80% decrease in decorin expression (219). The latter finding concurs with results from Li and Velleman (220) whom

also reported decreased decorin expression in response to TGF-β1 in both the endomysium and perimysium within the *pectoral major* muscle during chicken embryonic development.

Another study investigating decorin expression and more specifically decorin splice variants, reported the existence of two variants in human mesangial cells (HMC)(221). The expression of these decorin variants were shown to be affected by different concentrations of glucose. Furthermore, the changes in environment dictated the initiation site of the transcript, as decorin possesses two promoter sites (P1 and P2). Both these promoter sites are situated upstream of the first exon, hence after splicing both transcripts produces the same protein (359 aa)(221). The significance of this is likely related to the environmental factors activating gene transcription acting through different pathways.

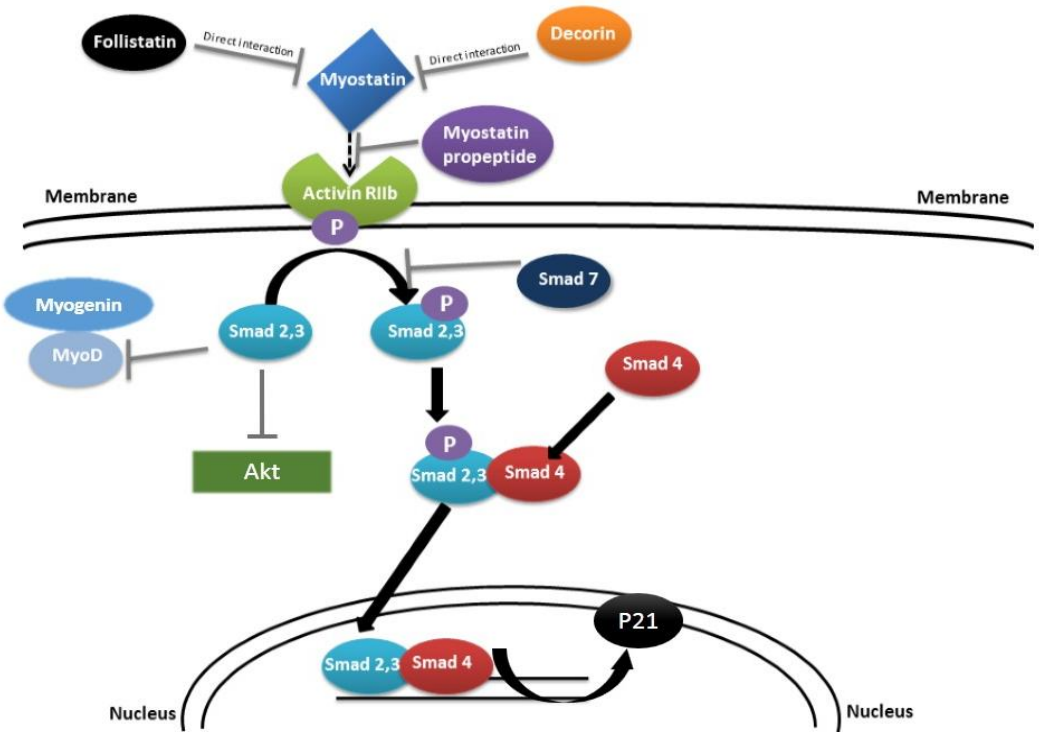


Figure 1.9 Myostatin pathway including regulatory proteins – Shows the binding of myostatin to the ActivinR IIb on the cell membrane and the initiation of the Smad pathway, which are able to inhibit several other factors. The Smad 2/3 and Smad4 transclocation to the nucleus results in the upregulation of p21. The regulatory proteins affect myostatin at different points; with decorin, myostatin propeptide and follistatin being able to influence the binding of myostatin to the receptor, while Smad 7 is responsible for intracellular inhibition by affecting the phosphorylation of the R-Smads.

1.4 Rationale

Although many studies have reported the myostatin response to exercise (162,165,166,222), focusing on endurance and resistance training, these studies did not place a lot of emphasis on the regulatory factors that have an influence on myostatin. These are essential factors to take into account when investigating myostatin or its signalling pathways since they could make the concentration of myostatin alone less relevant.

The reputation of myostatin as a negative regulator of skeletal muscle is firmly established by knock-out models or naturally occurring mutations (143,145). However, the effects of follistatin as a positive regulator of muscle mass have also been made very clear in a study over-expressing these protein in mice (203). Smad7 is also among the regulators of response to myostatin and has been reported to enhance myogenesis (223) and also to participate in the autoregulation of myostatin (186). Recently decorin was added to the group of factors with a regulatory role in relation to myostatin (191) and follow up research has proved the positive effect that decorin over-expression has on myoblasts by promoting both proliferation and differentiation (218).

There are numerous reported data emphasizing the major effects that these myostatin regulatory factors can have on skeletal muscle resulting in significant increases in muscle mass, by various mechanisms. This calls for the focus to be shifted or spread to include these factors in research pertaining to myostatin. Furthermore, the research previously done on these factors are mainly in rodent or cell models, as these are easier to manipulate. However since the functions of these factors are mostly characterized it is suggested to move on to a more complex and applicable model, humans. A list of human studies relating to myostatin and known myostatin interacting factors is provided in Tables 1.1 and 1.2. Additional studies such as the ones performed during the course of this thesis will enable a better understanding of the natural *in vivo* responses of skeletal muscle to interventions, such as exercise.

The use of eccentric exercise to investigate muscle regeneration would be the best option, as this form of exercise is made up of a stretch-shortening cycle (224,225) which introduces an eccentric muscle contraction to the exercise. Eccentric exercise causes disruption of muscle fibres (23,29,30); this damage initiates muscle regeneration with activation of satellite cells (226) and increased proteins synthesis leading to hypertrophy (227). This would suggest that myostatin would be tightly regulated in this period of time during early muscle regeneration, with the introduction of follistatin and decorin as direct myostatin regulators (175,191,218).

Table 1:1 Summary of human studies investigating myostatin in relation to exercise

1RM – 1 repetition maximum, RT – Resistance training, AE – Aerobic exercise, MB – Muscle biopsy, BD – Blood draw

(Ref) year	Subjects	Sex	Exercise protocol	Sample time points	Results
(228) **2007**	Untrained Young (27.0 years ± 10.1) Exercise group (n=9 Control group (n=6)	M	• Exercise group – 6 sets of 15 reps maximal eccentric contractions on dynamometer for 6 weeks • Control group – no exercise	• Control MB + BD 1 week before study • MB + BD 3 days into study • MB + BD 24 h after final exercise session	↓ MSTN mRNA throughout study compared to control
(229) **2009**	Young (21.5 ± 2.9 y) Recreationally active (n=13)	M	• 1st bout: 4 sets of 18-20 reps at 60-65% 1RM • 2nd bout: 4 sets of 8-10 reps at 80-85% 1RM • Single leg isotonic extensions	• MB 30 min, 2 h and 6 h post	↓ MSTN mRNA post exercise
(170) **2010**	Young (23.42±2.2 y) 3 groups: • Control (n=8) • Resist. + placebo (n=8) • Resist. + creatine (n=8)	M	• 3 days per week for 8 weeks • Each session, 3 sets of 8-10 reps at 60-70% 1 RM whole body exercise – bench press, lat pull down, bicep curls, leg press, knee extensors and knee flexors	• Serum samples at baseline (after overnight fast) week 4 and week 8.	RT ↓ serum MSTN RT + Creatine greater ↓ serum MSTN
(230) **2011**	Active young (22 ±1.6y) – 3 groups Endurance group (n=7) Strength group (n=7) Control group (n=7)	M	• 3/week 12 weeks training • Endurance training – running 45 min with HR of 80% of aerobic/anaerobic threshold • Strength training – whole body, 3 sets at 70-80% of 1RM • Control	• MB 3-5 days before the start and after completion of training regime	No sig difference MSTN mRNA for any group
(231) **2012**	Chronic heart failure (n=24) divided into 2 groups- Exercise group (n=12) Sedentary group (n=12)	M	• Sedentary control group • Exercise group – first 3 weeks exercise 6/day for 5-20 min cycle at 25 W then increase by 25 W every 3 min • 12 weeks training	• MB at baseline and after 12 weeks exercise • BD at baseline and after exercise protocol	↓ MSTN mRNA and muscle protein in exercise group No sig change in serum MSTN
(232) **2012**	Old (67.8 ± 1.0 y) (n=20) Old (67.2 ± 1.5 y) (n=15)	M F	• 3 sets of 12 reps maximal unilateral knee extensions	• MB at baseline and 2 h after exercise	↓ MSTN mRNA post exercise
(233) **2012**	Active young (23 ± 2y) (n=9)	M	• 45 min aerobic one legged cycle ergometer followed by 4 x 7 maximal concentric-eccentric knee extensions 6h later – one leg AE +RT, other only RT	• MB at baseline and 3 h after RT	↓MSTN mRNA in AE +RT group ↓MSTN mRNA in RT after training

Table 1:2 Summary of studies investigating myostatin and factors (follistatin, activin receptor IIB (ActRIIb) or Smad7) included in present study, *RE – resistance exercise, EE – eccentric exercise, CE – concentric exercise, RT – resistance training, MB – Muscle biopsy, BD – Blood draw*

(Ref) year	Subjects	Sex	Exercise protocol	Sample time points	Results
(206) **2007**	• Young (28 ± 5y) (n=10) • Old (68 ± 6y) (n=11)	M	• Bout maximal single leg eccentric knee extensions • 6 sets x 12-16 maximal contractions	• MB at baseline and 24 hr after exercise	No change MSTN mRNA No change follistatin mRNA - In any group
(168) **2007**	• Untrained older men (62.3 ± 6.3y)(n=22) • Strength training group (n=11) • Control group (n=11)	M	• Single RE bout before and after strength training period • 5 sets of 10 reps leg press	• MB at 30 minutes, 1 hr and 48hr after RE bouts	↓ ActivinRIIB mRNA receptor ↓ MSTN mRNA
(234) **2009**	• Older (61.4 ± 4.2y) • Protein group (n=9) • Placebo group (n=9)	M	• RE = 5 sets x 10 reps leg press • RE + 15g Whey protein (before and after) • RE + Placebo	• MB at baseline/rest and 1hr and 48 hr after RE	↑Follistatin-related gene (FLRG) mRNA in RE + Whey at 48hr post ↓ MSTN mRNA in RE + placebo at 48hr post No changes in ActivinRIIB mRNA
(207) **2010**	• Young (25 ±1 y) • Eccentric group (n=6) • Concentric group (n=6)	F	• 7 sessions • Group 1 – Single leg maximal eccentric knee extensions (EE) • Group 2 – single leg isokenetic knee extensions (CE)	• 8 h after 1st session • 8 h after 7th session • Muscle biopsy	No change FST or MSTN mRNA
(235) **2012**	• Young • • Low intensity resistance training (20.3 ± 4.2y) (n=10) • Low intensity + blood restriction resistance training (20 ± 4.5y) (n=10) • High intensity resistance training (23.6 ± 6y) (n=9)	M	• 8 weeks, bilateral knee extensions • Low intensity (20% 1RM)(LI) • Low intensity + restricted blood flow using an air cuff decreasing blood flow by around 50% (LIR) • High intensity (80% 1RM)(HI)	• MB baseline (1 week before start) and MB 48 h after last session	↓MSTN mRNA in LIR and HI No changes in ActivinR IIB mRNA ↓ FST related proteins mRNA –all groups ↑Smad7 mRNA in LIR and HI
(236) **2013**	• Old (61.2 ± 4.1 years) (n=18) • Untrained young (26.0 ± 4.3 years) (n=21)	M	• 21 weeks RT knee extensions and leg press + upper body	• MB before and 1 week after the last training session	↑ MSTN mRNA in older RT group No sig diff in FLRG or ActivinRIIB in any group

1.4.1 Aims

1. To determine the existence of exercise-induced muscle damage by means of functional testing and serum analysis
2. To establish if splice variants of the decorin gene are present in human skeletal muscle and to quantify their expression in response to eccentric exercise along with immunoblot analysis for the decorin protein.
3. To quantify proteins involved in the myostatin pathway, including myostatin regulatory factors such as follistatin and decorin in response to eccentric exercise.
4. To quantify changes in expression of Smad proteins involved in myostatin signalling, including both the pro-signalling proteins (phosphorylated Smad2/3) and the signalling inhibitory proteins (Smad7).

1.4.2 Hypothesis

- Isometric force production will decrease immediately after an acute bout of unaccustomed eccentric exercise in subjects with average fitness
- Myostatin protein concentration will be decreased in the short period after an acute bout of plyometric exercise
- The Smad signalling cascade will not be activated during early stages of regeneration, concurring with myostatin concentration changes.
- Activin IIb membrane bound receptor would decrease in skeletal muscle shortly after exercise with lengthening contractions as result of membrane damage
- Regulatory factors affecting myostatin directly or interfering with the canonical pathway will be increased in early muscle regeneration after acute bout of eccentric exercise.
- Follistatin will be mostly affected by exercise, as it serves to regulate an array of growth inhibitory factors which will possible hinder protein synthesis and muscle regeneration
- Variant transcripts of decorin are present in human skeletal muscle and the expression patterns of the variants respond to exercise induced muscle damage

2 Chapter 2: Materials and Methods

2.1 Reagents list

Table 2:1 List of reagents used in the laboratory analysis along with the manufacturer and catalogue numbers

Reagents	Manufacturer	Cat. number
2-Mercaptoethanol	Sigma-Aldrich, USA	**M3148**
Acrylamide solution 40%	Sigma-Aldrich, USA	**A4058**
Ammonium persulfate–APS	Sigma-Aldrich, USA	**A3678**
Benzamidine	Sigma-Aldrich, USA	**135828**
Boric acid	Sigma-Aldrich, USA	**B6768**
Bovine Serum Albumin fraction V – BSA	Roche, USA	**10735078001**
Bromophenol Blue	Sigma-Aldrich, USA	**318744**
Chloroform	Sigma-Aldrich, USA	***C2432***
Enhanced chemiluminescence (ECL) – SuperSignal West Pico	Pierce – Thermo, USA	**34087**
Ethanol	Sigma-Aldrich, USA	***E7023***
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, USA	**E9884**
Glycerol	Merck- Calbiochem, USA	**356350**
Glycine	Sigma-Aldrich, USA	**G8898**
Isopentane	Merck Millipore, USA	**106056**
Isopropanol	Sigma-Aldrich, USA	***I9516***
Potassium chloride – KCl	Merck- Calbiochem, USA	**529552**
Leupeptin	Merck- Calbiochem, USA	**108976**
Methanol	Merck-Millipore, USA	**1060095000**
$MgCl_2$– Magnesium chloride	Merck- Calbiochem, USA	**442611**
***N,N,N′,N′*-Tetramethylethylenediamine (TEMED)**	Sigma-Aldrich, USA	**T7024**
Nonidet™ P-40	Sigma-Aldrich, USA	**74385**
PMSF- phenylmethylsulfonyl fluoride	Sigma-Aldrich, USA	**P7626**
Ponceau S	Sigma-Aldrich, USA	**P7170**
PUGNAc	Sigma-Aldrich, USA	**A7229**
Sodium acetate	Sigma-Aldrich, USA	**S2889**
Sodium chloride –NaCl	Merck- Calbiochem, USA	**567441**

Sodium deoxycholate	Sigma-Aldrich, USA	**30970**
Sodium Dodecyl Sulphate (SDS)	Sigma-Aldrich, USA	**L3771**
Sodium fluoride	Sigma-Aldrich, USA	**S7920**
Sodium hydroxide – NaOH	Merck- Calbiochem, USA	**567530**
Sodium Orthovanadate	Merck- Calbiochem, USA	**567540**
Sodium phosphate dibasic -Na2HPO4	Sigma-Aldrich, USA	**S7907**
Soybean trypsin inhibitor (SBTI)	Sigma-Aldrich, USA	**T9128**
Tris-base – Trizma base	Sigma-Aldrich, USA	**93349 FLUKA**
Triton X-100	Sigma-Aldrich, USA	**93443**
Tween 20™	Sigma-Aldrich, USA	**P7949**
Hydrochloric acid 32% - HCl	Merck- Millipore, USA	**1003192511**

2.2 Ethics statement

This study was approved by Sub-Committee C of the Research Committee of the University of Stellenbosch, ethics reference number: N12/08/051 (Approval letter, Appendix). This ensured that the study was conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

2.3 Initial subject recruitment

Healthy adult males (n=25) aged between 20-25 years of age were recruited for this study via the use of poster advertisements placed within Stellenbosch university buildings, oral presentations to undergraduate students and social media i.e. Facebook (www.facebook.com). Initial selection criteria for participants involved the following –

2.4 Inclusion requirements

- Participants had to be physical active, but only participating in a light exercising regime with of frequency of 0-2 exercise sessions per week. The exercise regime which participants engaged in must not have included any form of eccentric exercise, for example downhill running or jumping squats.

2.5 Exclusion parameters

- Individuals which are highly trained elite athletes
- Individuals suffering from type 1 diabetes
- Individuals chronically treated by any corticosteroid-containing medication.

Participants who satisfied the above criteria were then invited to the exercise physiology research labs at Stellenbosch University whereby the specific details of the study and requirements of participants was provided. Informed consent from participants was then obtained.

2.6 Study design

From the initial recruitment of 25 subjects, only 15 were chosen to continue. Participants visited the exercise physiology laboratory at Stellenbosch University on 10 separate occasions over the course of (8 weeks) in order for all physiological measurements to be taken. A schematic of what each of the ten visits entailed is provided in Figure 2.1 while a detailed description of protocols used in collection of tissue and blood samples is provided below.

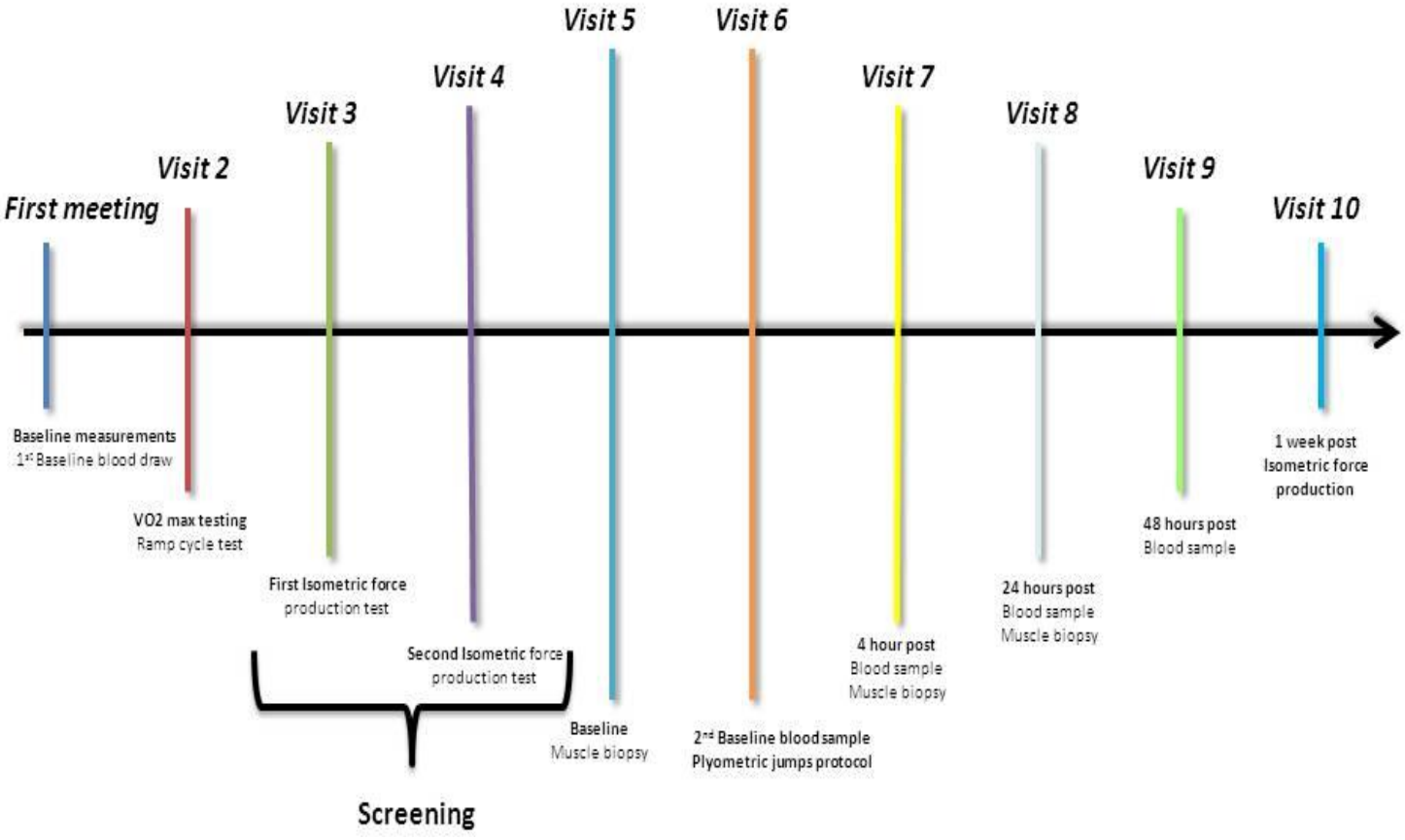


Figure 2.1 Time line scheme of the research trial

Visit 1 – Height, weight and blood pressure measurements were obtained for each participant. A blood sample was also obtained that would provide information on the baseline levels of creatine kinase for each participant. The protocol for each blood draw was as follows:

- Participants were placed in a supine position
- An 21 gauge needle (PrecisionGlide™, BD Vacutainer, UK) was inserted into the antecubital vein of the participants
- 5ml of blood was drawn into a serum separating tube (SST II Advance, BD Vacutainer, UK)
- Post blood draw serum separating tubes were inverted 5-6 times in accordance with manufacturers guidelines
- Serum samples were sent to Pathcare laboratories (MediClinic, Stellenbosch, RSA) and serum creatine kinase concentration was quantified by means of an activity assay using a SYNCHRON LX system (Beckman Coulter, USA) that utilises the chemical reaction outline in figure 2.2 to ascertain CK levels.

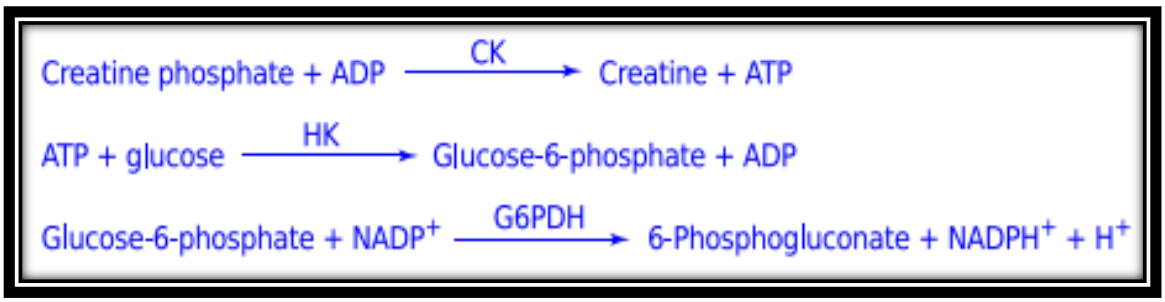


Figure 2.2 The chemical reaction occurring during the quantification analysis of creatine kinase- [Adapted from Chemistry information sheet A18477AK, Creatine Kinase, Synchron systems chemistry information sheet, Beckman Coulter

Increased CK levels will result in increased production of ATP serving as a substrate for two coupled enzyme reactions with hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH). These enzymatic reactions lead to an increase in reduced β-nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH levels are then measured at 340 nm and thus giving an indirect measure of CK levels.

Visit 2: VO_2 max testing on a cycle ergometer (VeloTron, Computrainer, USA) was conducted on the second visit (see Figure 2.3 for image of VO_2max testing setup). Participants were asked not to have engaged in any strenuous physical activity for at least 2-3 day before testing. Prior to commencement of test VO_2 max testing, the procedure was verbally explained. The VO_2 max test protocol used was as follows.

- Participants arrived at the Department of Physiological Sciences' in-house laboratory at their allocated time slot.

- 5 minutes of dynamic warm-up on a stationary bicycle (VeloTron, Computrainer, USA) was performed followed by static stretching of the quadriceps and hamstring muscle groups. During the warm up period a verbal explanation was provided to participants with regards the need for them to cycle till they reach complete exhaustion in order to obtain an accurate assessment of the VO_2 max state.
- Oxygen masks (Vmask, Hans Rudolph, USA) were fitted and tested for air leaks prior to commencement of test (Figure 2.4).
- A metabolic analysis system (Oxycon Pro, Jaeger, Germany) was utilised for breath-by-breath gas analysis.
- A continuous ramp protocol was conducted on the cycle ergometer, starting with a workload of 100 watt and increasing it by 20 watts every minute. Participants continued to pedal as the resistance increased until they were unable to maintain a cadence of >=70. The mask in association with the metabolic analysis system recorded the amount of O_2 used by the participant. The values at which the VO_2/kg (ml/min/kg) peaked and plateau were used to calculate the respective VO_2 max.

Figure 2.3 Cycle ergometer setup for VO2 max testing

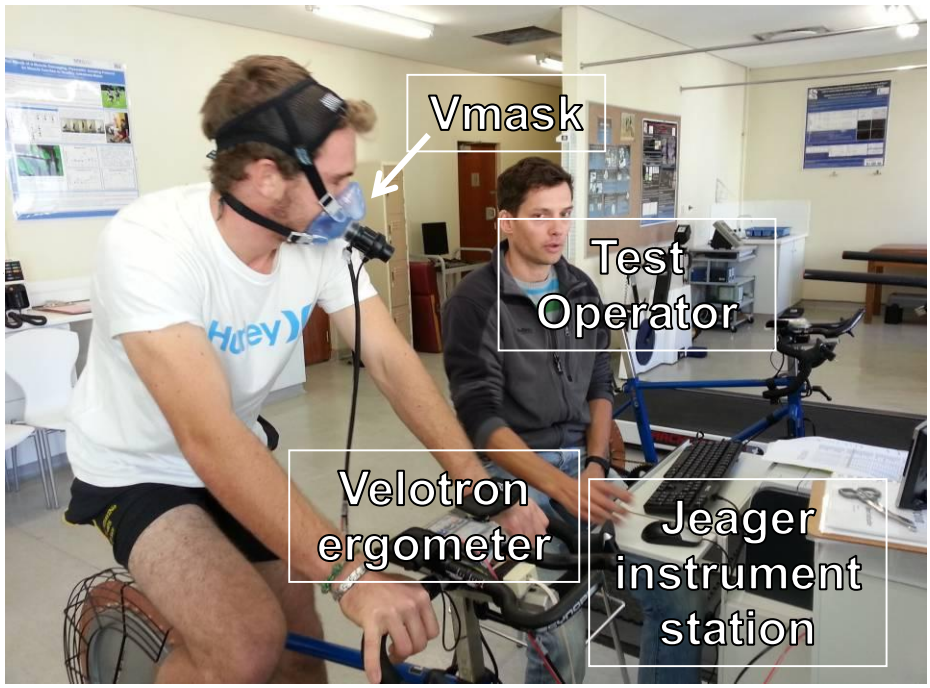


Figure 2.4 Subject setup and mask fitting for VO2 max testing – shows the setup procedure for the subjects before the start of the starting the test. The mask fitted and all systems were calibrated before connecting the final tube connecting the mask with the Oxycon Pro

Visit 3: Participants performed their first attempt at maximal isometric force production. The purpose of this visit was to familiarize the participants with the in-house isometric force chair (see Figure 2.5) for image of force chair) and testing procedure. The isometric force chair is similar in design to a standard leg extension machine that is present in most gyms. Our in house chair was designed by the Engineering faculty, University of Stellenbosch. Protocol for isometric force testing was as follows

Figure 2.5 Isometric force testing apparatus: Shows the chair used in the testing. The screen is connected to a computer and is used to visualize the in-test results. The crossbar was setup at 30^{o} from the horizontal axis

- Participants performed a five minute warm up that consisted of a slow jog on a treadmill (Runrace, Technogym, Italy) followed by a combination of static and dynamic stretching focusing particularly on the quadriceps and hamstring muscle groups.
- Participants were then seated in the chair with the crook of their knee against the seat and their back comfortably against the back rest. The crossbar was adjusted to the *popliteal fossa* (crook-of-the-feet) while their feet were under dorsal flexion. The position of the backrest and crossbar were noted and entered into the custom computer software designed for the chair.
- The geometric measurements of the knee-ankle, knee-floor, ankle-floor and knee- front-of-seat measurements shown in Figure 2.6. The hinge angle was set at 30° from horizontal axis, to ensure that the *quadriceps* will be the main contributors to the production of isometric force during the test. This angle was consistent throughout the study.

Figure 2.6 Subjects setup concerning geometric measurements

- Once participant set-up in the chair was completed, they were strapped in to ensure their *gluteus maximus* and lower back were stably positioned against the seat throughout the test (Figure 2.7). Participants were initially asked to lightly push against the crossbar and then remove their feet from the crossbar completely so that a “zero force reading” could be

taken. The protocol was verbally explained to participants followed by the completion of one practice attempt.

Figure 2.7 Subject setup for isometric testing – illustrates the setup, including the strap-in of the participant to ensure the gluteus maximus and lower back is kept against the seat. The figure also shows the crossbar at the popliteal fossa and the computer stat

- Participants subsequently performed three maximal efforts that involved participants pushing their ankles into the crossbar cushion by flexion of the quadriceps muscle group. Each of the repetitions was held for 10 seconds, as an isometric contraction, and 20 seconds rest was allowed between repetitions. Verbal motivation was used during the testing to encourage maximal force output. Participants also were provided with a real time graphical view of their force output on a computer screen adjacent to the testing chair.

Visit 4: Participants performed their 2nd attempt at the maximal isometric force production test.

Visit 5: Baseline muscle biopsies were obtained by a qualified medical doctor from participants. Protocol used in obtaining biopsies was as follows

- Biopsy area on the *vastus lateralis* was initially cleaned with 70% alcohol.
- Subsequently a local anaesthetic (2% Lignocaine HCL m/v) and 1: 80 000 nor-adrenaline (Xylotox, Adcock Ingram Ltd, Johannesburg, RSA) was injected around the sterilised site on the *vastus lateralis* of the participant.

- A sterile surgical blade was used to make a small incision before a5mm trephine biopsy needle (Bergstrom biopsy needle, STILLE, Sweden) with assisted suction extracted the skeletal muscle tissue.
- Post biopsy the skeletal muscle tissue was carefully removed from the biopsy needle and placed into cryogenic vials (Corning Incorporated, Mexico) – one for mRNA isolation and one for protein isolation.
- Cryogenic tubes were then placed into liquid nitrogen cooled isopentane (Merck Millipore USA) and subsequently stored at -80c until analysis.

Note *- Biopsies were taken from alternate legs. In total 3 biopsies were obtained from each participant. For the leg which was biopsied twice the second biopsy was taken 3cm from the site of first biopsy*.

Visit 6: Participants arrived at the exercise laboratory at 13:00 for their pre-exercise/2nd baseline blood draw and performance of the plyometric exercises. Protocol used for the plyometric jumps was as follows.

- Participants performed a warm up that consisted of 10 minutes of light jogging and static stretching for the hamstrings and quadricep muscle groups.
- Following the warm-up, the proper technique for performing the plyometric jumps was explained (see Figure 2.8 for diagrammatic illustration of plyometric jumping technique).
- Participants were then given a piece of chalk and asked to perform three preliminary maximal jumps. At the top of each jump the participant marked the wall with the chalk.
- The height of the preliminary maximum jumps was measured from the floor and 90% of the measured height was calculated and used as the target height for the subsequent exercise protocol that consisted of 10 sets of 10 plyometric jump repetitions (100 reps total) with a 60 second rest between sets.
- Plyometric jumps were visually monitored to confirm that the participant reached or exceed the target height.

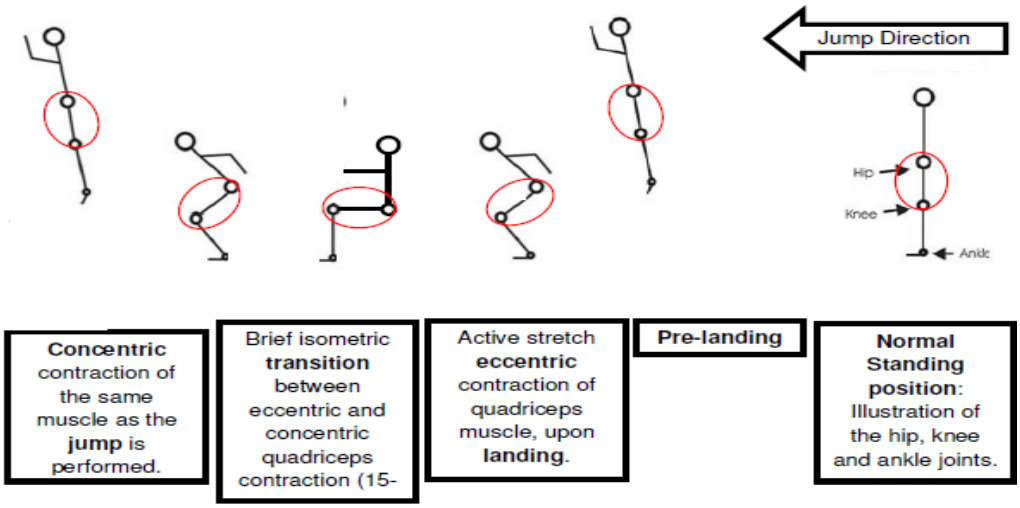


Figure 2.8 Technique sequence for plyometric jumps: Shows the proper technique for the execution of the plyometric vertical jump [adapted from the MSc Thesis of A. Isaacs, 2012]

Visit 7: Participants returned to the exercise physiology lab at Stellenbosch University 4 h (17:00) post completion of the plyometric jump protocol in order for biopsies and blood draws to be taken.

Visit 8: Participants arrived at the exercise physiology laboratory 24 h post performance of their plyometric jumps. Biopsies and blood draws were taken at this time point.

Visit 9: Participants returned to the exercise physiology laboratory 48 h post completion of their plyometric jumps in order for a blood draw to be taken at this time point

Visit 10: Participants completed a maximal isometric force production test 1 week post completion of the plyometric jump.

2.7 Western Blot

2.7.1 Biopsy homogenization

Skeletal muscle biopsies were homogenized with the Bullet Blender® Storm 5 (Next Advance, Inc. USA) tube containing 100 mg of beads and 1 ml lysis buffer (Tris 50 mM, EDTA 100 mM, Benzamidine 1 mM, sodium fluoride 1 mM, PUGNAc 1 mM, Triton X-100 1% (v/v), NP-40 1% (v/v), sodium deoxycholate 0.25% (m/v), leupeptin 0.1% (m/v), SBTI 0.4% (m/v), sodium orthovanadate 100 mM and 1 mM PMSF).

2.7.2 Protein concentration measurements

Protein concentrations of the samples were measured using a commercial Bicinchoninic acid (BCA) kit (BCA protein assay, Thermo Fischer Scientific, USA) and using Bovine serum albumin (BSA) (Bovine Serum Albumin Fraction V, Roche, USA) as standards, ranging from 1 mg/ml to 8 mg/ml. The assay was performed on a 96-well plate (PS microplate, Greiner Bio-One, USA) with 200 uL BCA added to 10 uL of the sample or standards. All samples and standards were loaded in triplicate. Protein concentration was measured at 595 nm via a plate reader (EL-500 Universal Microplate reader, Bio-Tek Instruments Inc, USA). Concentrations of the samples were calculated using the standard curve as reference.

2.7.3 ABC chondroitinase treatment for decorin probing

Prior to the immunoblotting for decorin, the samples were treated with Chondroitinase ABC from *Proteus vulgaris* (C3667, Sigma-Aldrich, USA) to remove the polysaccharide side chains attached to the decorin core protein. Samples (30 µg) were incubated at 37˚C for 6 hours in a buffer solution (BSA 0.02%,Tris-HCl 50 mM, sodium acetate 60 mM, pH 8.0) with 36 mU Chondroitinase ABC enzyme. Following the 6 hour incubation period, Laemmli sample buffer (237) 0.0625M Tris-HCl (p 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% Bromophenol blue) was added and the samples were boiled at 95˚C for 5 minutes and the proteins were separated on a 15% acrylamide gel.

2.7.4 Polyacrylamide Electrophoresis and transfer

Polycrylamide gels consisting of a 15% separating gel (0.39 M Tris-HCl (pH 8.8), 15% Acrylamide, 0.1% SDS, 0.1% APS, TEMED) and a 4% stacking gel (4% Acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 0.1% APS, TEMED) were utilised for electrophoresis of protein samples. Gels were placed in a Mini Protean Tetra system (Bio-Rad, USA), submersed in running buffer (3.46 mM SDS, 25.01 mM Tris base, 191.95 mM Glycine) and electrophoresed at 120 volts until the blue dye

present in the Laemmli solution had reached the bottom of the gel. Post electrophoresis gels were transferred onto a nitrocellulose (GE Healthcare, Life science, RPN 3032D, UK) membrane via a Turbo-blot transfer system (Bio-Rad, USA). Transfers were performed for 30 min at 25 volts and 1 amp. A Precision Plus Protein™ Kaleidoscope™ marker, (Standards #161-0375, Bio-Rad, USA) was used obtain information on protein sizes. Each gel contained at least 2 complete sets of samples from 2 participants' i.e. baseline, 4 h- and 24 h post exercise. Also present in each gel was a reference sample, consisting of extra muscle sample that was homogenized, that enabled comparison of protein expression between gels.

2.7.5 Immunodetection protocol

Post transfer blots were stained with Ponceau S for 5 minutes, followed by 3 x washes with dH2O (until the background is clear white). The ponceau stained membrane was then captured using on a ChemidocMP imaging system (Bio-Rad, USA) that was supported by (Image lab software 4.0, Bio-Rad, USA). Images confirmed successful transfer of the proteins on the membrane. Post ponceau staining membranes were washed in 1x TBS-T (49.8 mM Tris-HCl (pH 7.6), 149.8 mM NaCl, 0.1% Tween-20®) and blocked for 1 h with either semi skimmed milk or BSA (see Table 2.2 for information on specific blocking used for each antibody). Post blocking membranes were washed 1 x 10 minutes followed by 2x 5 minutes with 1X TBS-T. Subsequently membranes were then incubated with primary antibodies as outlined in Table 2.2. Post primary antibody incubation membranes were washed 1 x 10 minutes and 2 x 5 minutes with 1X TBS-T followed by a 1h incubation at room temperature with the required horseradish peroxidase (HRP)-linked secondary antibody. Specific secondary antibodies used for each primary antibody are provided in Table 2.2. Post-secondary antibody incubation, membranes were washed 3 x 10 minutes with TBS-T followed by addition of enhanced chemiluminescence (ECL). Membranes were subsequently imaged using the Chemidoc MP (BioRad, USA) that was supported with Image lab software 4.0 by Biorad. Immunoreactive proteins were subsequently quantified using the software and were normalised against total ponceau staining. These normalised values were then divided by the value of the normalised value of the reference sample which was present on all membranes and thus enabled comparison of protein values between membranes.

Table 2:2 Antibody optimized conditions for immunoblotting: Shows a summary of all the antibody concentrations, blocking substance and concentration, incubation times and secondary concentrations used during immunoblotting.

Primary Antibody	*Blocking with primary Ab*	*Dilution*	*Blocking*	*Primary Ab Incubation time*	*Secondary Antibody*	*Dilution*	*2° Ab Incubation time*
p-SMAD 2/3 (Goat)	Non	1: 1000	3 % BSA	Overnight @ 4^0C	Sigma	1: 15000	1 hour at RT
SMAD 6/7 (Goat)	1% Milk	1: 1000	5 % milk	Overnight @ 4^0C	Sigma	1: 10 000	1 hour at RT
Myostatin/GDF-8 (Rabbit)	Non	1: 1000	3 % milk	Overnight @ 4^0C	Cell signalling	1: 15000	1 hour at RT
Decorin (Mouse)	Non	1:500	3% milk	Overnight @ 4^0C	Abcam	1: 10 000	1 hour at RT
Activin RIIB (Goat)	non	1: 1000	5% milk	Overnight @ 4^0C	Sigma	1: 15 000	1 hour at RT
Follistatin (Rabbit)	non	1: 1000	3% milk	Overnight @ 4^0C	Cell signalling	1: 20 000	1 hour at RT

2.8 RNA Analysis

2.8.1 RNA isolation

Approximately 20 mg of skeletal muscle biopsy material was homogenized in 1 ml of TriPure (11667157001, TriPure Isolation Reagent, Roche, Switzerland) using specialized beads in association with the Bullet Blender® Storm 5 (Next Advance, Inc. USA), and according to the manufacturer's instructions for skeletal muscle. Total RNA was isolated from the Tripure solution according to the manufacturer's guidelines, which briefly entailed the following steps:

Step 1- Chloroform was mixed with the TriPure solution that contained the homogenised skeletal muscle lysate in a 1:5 ratio (cholorform:Tripure) and left at room temperature for 10 minutes.

Step 2 - Samples were then centrifuged at 12 000x g for 15 minutes, resulting in a 3 tier physical separation of the RNA (top layer), DNA (middle layer) and proteins (bottom layer in the lysate). The top layer containing the RNA was carefully removed and transferred to a new RNAse free tube (Ambion, USA).

Step 3 - Isopropanol was then added in an equal volume to the removed RNA. Samples were inverted several times to mix the two solutions and then left at room temperature for 10 minutes.

Step 4- Samples were centrifuged at 12 000x g for 10 minutes, resulting in the formation of an RNA containing pellet at the bottom/side of the tube. Supernatant was removed from the tube and 400 µL 70% ethanol was added to the tube to wash the pellet.

Step 5- Samples were centrifuged again at 8 500x g for 6 minutes. Following the centrifugation, the supernatant was aspirated and the tubes were left open at room temperature for 5-10 minutes to remove any residual ethanol.

Step 6 -The isolated RNA was re-suspended in TE buffer (10 mM Tris (pH8), 1 mM EDTA)(AM 9847, Ambion, USA) and stored at -20^{o} C until use.

To check for quality of the RNA 500 ng of RNA was run on an agarose gel and thereafter visualized on the ChemidocMP (BioRad, USA) to evaluate if the 18s and 28s ribosome were intact as a quality control step.

2.8.2 Reverse Transcription

Concentration of the isolated RNA was determined by a NanoDrop Mini (Thermo Fischer Scientific, USA) and 1 µg of the isolated RNA was treated with DNAse according to the manufacturer's guidelines (#04716728001, DNase I recombinant, RNase-free, Roche, Switzerland) to remove any residual DNA carryover from the isolation process.

Subsequently a commercial reverse transcription kit (Transcriptor first strand cDNA synthesis, #04896866001, Roche, Switzerland) was used to reverse transcribe the DNAse treated RNA into cDNA with random hexamers in accordance with the manufacturers'guidelines.

2.8.3 Primer Design

The NCBI database (www.ncbi.nlm.nih.gov) was used to obtain sequence information pertaining to the human decorin mRNA transcripts A1 (NM_001920.3) and A2 (NM_133503.2) and GAPDH. Sequence present in Exons 1 and 2 of decorin A1 were used to design decorin A1 specific primers as these two exons are only in dercorin A1. Primers specific for decorin A2 were designed using sequence present in exons 1 and 2 of decorin A2 as these two exons of only present in decorin A2. All of the a fore mentioned sequences fordecorin A1, A2 and GAPDH were inputted into the free online primer design tool OligoPerfect™ available at http://tools.lifetechnologies.com. Primer sequences for DecorinA1, A2 and GAPDH are provided in Table 2.3

Table 2:3 Semi-Quantitative mRNA analysis primer information: Shows the details pertaining to the primers that was designed and custom made for the analysis of A1 and A2 decorin mRNA variants in human skeletal muscle. Furthermore, the table also include information of the GAPDH primer used to confirm cDNA loading in all samples.

Primer sets (Sigma-Aldrich, USA)	Sequence	Amplicon size	Bp	Tm (C^0)	GC %
Dec A1 Forward	ATTGTGTTCATTGAGGGAAACC	260	22	63.7	40.9
Dec A1 Reverse	AGCAGAAGGAGGATGATAGTGG		22	63.4	50
Dec A2 Forward	ATCGTCTAGTGAGGGACAGACC	260	22	63.4	54.5
Dec A2 Reverse	GCAAGCAGAAGGAGGATGATAG		22	64.0	50
GAPDH Forward	AATCCCATCACCATCTTCCA	716	20	56.17	45
GAPDH Reverse	TGACAAAGTGGTCGTTGAGG		20	58.05	50

2.8.4 Semi quantitative PCR

Total volume of each PCR was 25 µL that consisted of the following 2 µL of cDNA (50 ng), 1.25 uL forward and reverse primers (primer concentration per reaction being 1 uM), 8 uL nuclease free H_20 and 12.5 uL of the PCR mastermix (#04710444011, FastStartPCR Master, Roche, Switzerland). All PCR experiments were carried out on a thermocycler (2720, AppliedBiosystem, USA) in accordance with PCR mastermix manufacturers' guidelines. Briefly the steps for each PCR was as follows

Step 1: 5 minutes at 95^0C

Step 2: 15 seconds at 95^0C

Step 3: 30 seconds at 56^0C

Step 4: 1 minute at 72^0C

} 40 cycles

Post PCR samples were mixed with 6 µL loading buffer (75% v/v glycerol, 0.02% w/V bromophenol blue, 10 mM Tris Base, 1 mM EDTA, 0.2% w/v SDS) and loaded onto a 1.5% agarose gel containing sybr safe DNA gel stain (#533102, Invitrogen, Life Technologies, USA) at 1X concentration. The samples were electrophoresed using a Gel XL Ultra electrophoresis system (Labnet International, USA) and 1x TBE running buffer (89 mM Tris Base, 89 mM Boric Acid, and 3 mM EDTA). Gels were electrophoresed at 50 V for about an hour or until the bromophenol blue dye had reached the halfway point of the gel. A 100 bp ladder (15628-050, 100 bp DNA ladder, Invitrogen™, Life Technologies, USA) was run alongside the samples for visualization of the product size. The gels were visualized on a Chemidoc MP (Bio-Rad, USA) and analysed by using Image Lab software 4.0 (Bio-Rad, USA). To confirm identity of detected amplicons, bands of the correct base pair length were excised from the gel under an UV-light. Subsequently gel pieces were sent to the Central Analytical Facility (University of Stellenbosch, Stellenbosch, RSA) for sequencing. Results obtained from sequence reaction were then aligned to the NCBI human genome and transcript database via use of the basic local alignment search tool software available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.8.5 Quantitative PCR (qPCR)

TaqMan® probe chemistry was used for (qPCR). TaqMan® probes used for detection of decorin were Hs01072200_m1 which could detect decorin transcripts A1 (NM_001920.3) and A2 (NM_133503.2) while TaqMan®Hs01075781_m1 probe could detect decorin transcript A2 (see Table 2.4).

Each reaction for qPCR consisted of the following – 10 uL TaqMan® gene expression master mix, 1uL TaqMan® probe, 1.2uL cDNA (30 ng) and 7.8 uL of nuclease free H_20 to give a total volume of 20 uL. All samples were run in duplicate in 96 well qPCR plates (#4483352, MicroAmp® EnduraPlate™ Optical 96-Well Clear Reaction Plates, Life Technologies, USA) on a StepOnePlus™ Real-Time PCR system (4376600, Life Technologies, USA) with the accompanying StepOne™ Software v2.2. The settings for the thermocycler was as follows: 2 minutes at 50^0C followed by 10 minutes at 95^0C, then 40 cycles of 15 seconds at 95^0C followed by 1 minute at 60^0C.

Table 2:4 Information on the probes used in the quantification of the genes with real-time PCR

Cat probe	Target transcripts	Exon boundaries	Amplicon length
Hs01072200_m1	NM_001920.3Decorin A1	4-5	102
	NM_133503.2Decorin A2		
Hs01075781_m1	NM_133503.2Decorin A2	1-2	108

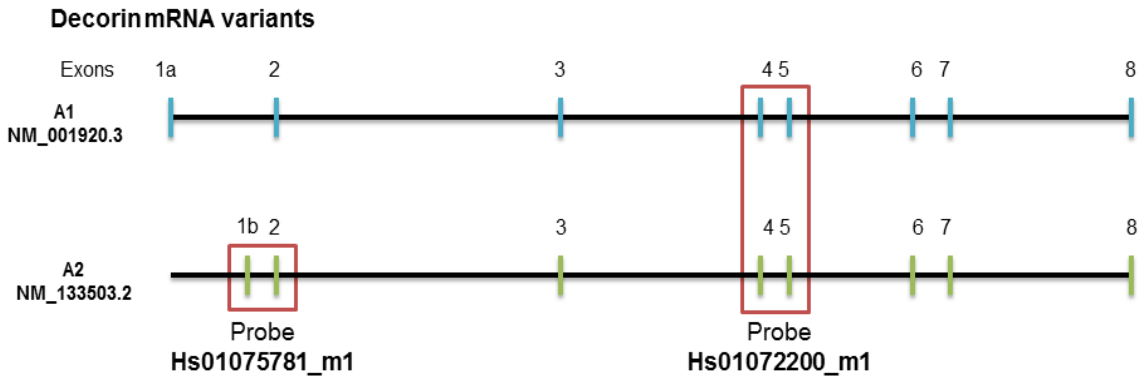


Figure 2.9 RT-PCR amplification regions for specific probes– Shows the exon map for the decorin [Homo sapiens] gene with different initiation sites (1a and 1b) for the two mRNA variants of interest. The figure also shows the exon boundaries for the specific probe

2.8.6 Quantification of data

Expression levels were quantified using the comparative $2^{-\Delta\Delta CT}$ calculation method (238) using beta-2-microglobulin as the house keeping gene. TaqMan® probe used for B2M was Hs00984230. A reference sample, which was one of the samples from another participant, was also included on each plate and all samples were normalised to this.

2.9 Statistical Analysis

All statistical analysis was performed with STATISTICA software 11.0 (StatSoftInc, USA). Normal probability plots were constructed for all data to assess the normality assumptions of the data. Mixed models repeated measures ANOVA and Fisher LSD post-test was perform on all data to conclude significant differences between mean values of different time points. Standard deviations are shown on bar graphs and statistical significance are indicated when $p \leq 0.05$.

3 Chapter 3: Results

3.1 Baseline data

Anthropometric measurements, age profile and VO_2 max values of participants whom completed this study are provided in Table 3.1.

Table 3:1 Summary of the subject's baseline characterization values: Data are shown as mean values ± standard deviation (SD). Values reported are from the selected group of participants continuing with the study after the screening sessions.

	Study (n=15)
Age (years)	21 ± 2.56
Height (cm)	179.17 ± 3.44
Weight (kg)	77 ± 15.69
Body mass index (BMI) (kg/m^2)	23.93 ± 4.46
VO2 max (ml/kg/min)	44.1 ± 4.38

3.2 Effect of an acute bout of plyometric jumping on maximal isometric force output

In order to ascertain the effects of an acute bout of plyometric jumping on skeletal muscle, participants maximal isometric force production of the quadriceps muscle group was measured via a custom built isometric force chair. Participants were assessed pre performance of plyometric jumps, immediately after completion of the plyometric jumping protocol and 1 week after completion of plyometric jumps. A significant ($p = 0.045$) decrease in force production was observed immediately after (12.02 N/kg ± 1.73 SD) completion of plyometric jumps compared to baseline values (13.08 N/kg ± 2.49 SD) and 1 week (13.38 N/kg ± 2.24 SD). The significant reduction (10%) in force output immediately after completion of the plyometric jumping protocol indicates that the protocol did indeed affect voluntary force production. While the return to baseline isometric force output values 1 week post completion of the protocol demonstrates that 1 week is sufficient for skeletal muscle of participants to recover their force production capabilities after a plyometric jumping intervention

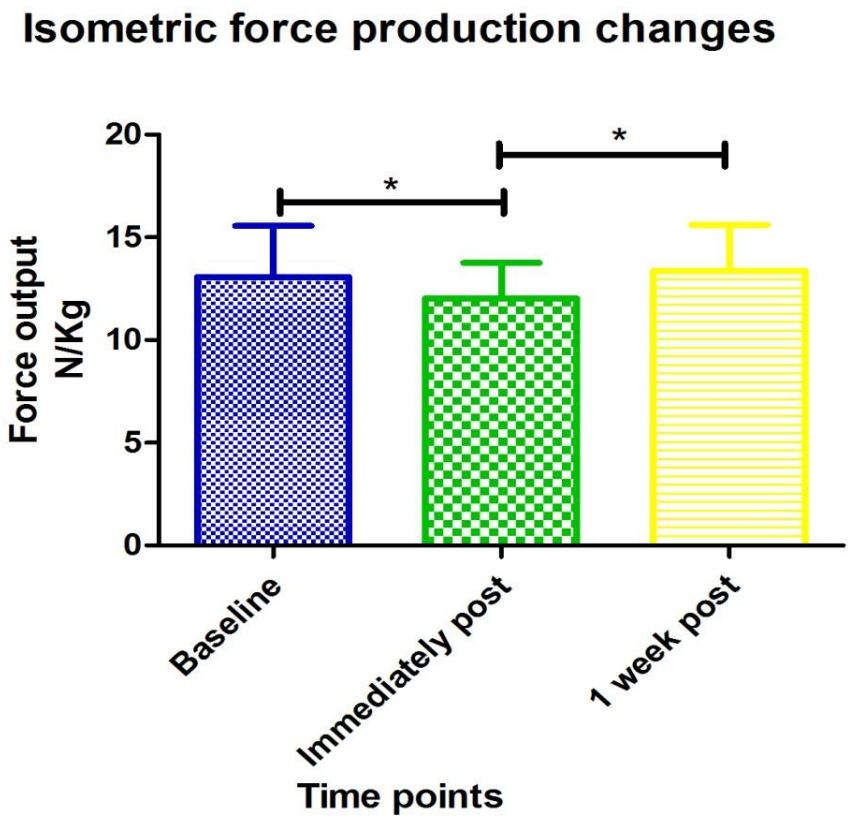


Figure 3.1 Maximal isometric force production changes over time – Error bars in the graph represent standard deviation (SD). (* = $p < 0.05$)(n=15)

3.3 Effect of an acute bout of plyometric jumping on serum creatine kinase levels

Participant's serum creatine kinase levels were measured at baseline and at 4, 24 and 48 h post completion of the plyometric jumping protocol. A significant increase ($p < 0.05$) in serum creatine kinase levels was observed at all-time points assessed post completion of the plyometric jumping protocol compared to baseline levels. The highest creatine kinase levels were observed at 24 hours (626.92 u/L ±496.81 SD) with slightly lower levels being present at 4- (422.69 u/L ± 429.17 SD) and 48 h (452.08 u/L ± 172.46 SD) post completion of the plyometric jumping protocol.

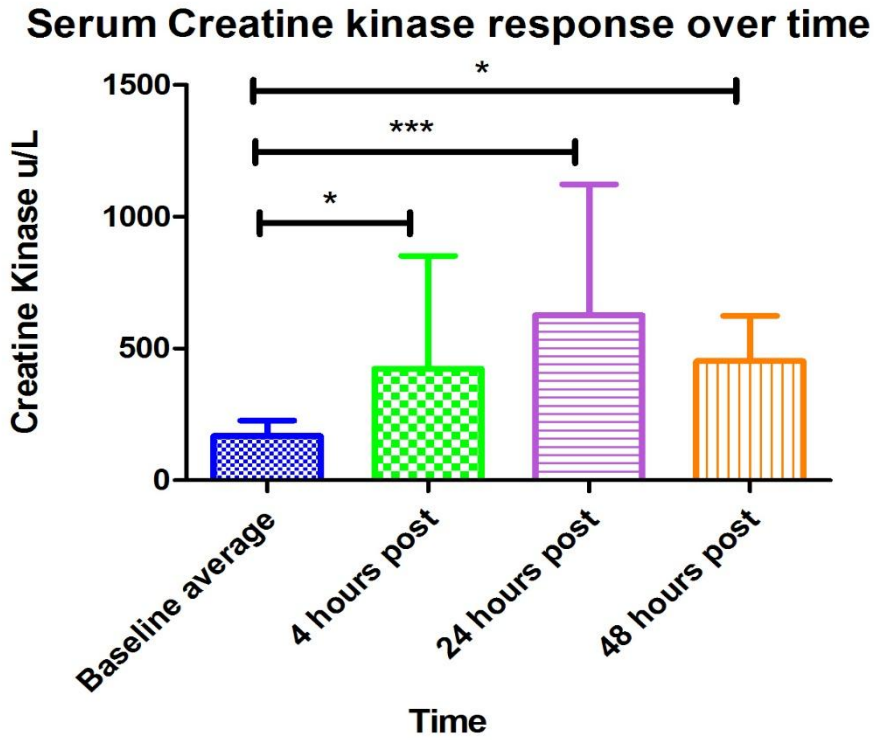


Figure 3.2 Serum creatine kinase response over time – Error bars represent standard deviation (SD)(n=15). * p<0.05, ** p<0.01, *** p<0.001

3.4 Molecular adaptation of skeletal muscle in response to an acute bout of plyometric jumping

3.4.1 Myostatin protein expression

Myostatin protein expression values were assessed via SDS PAGE and immunodetection. A commercial antibody directed towards a region spanning from the 300th amino acid to the C-terminal of myostatin was utilised (Anti-Myostatin antibody, ab98337, Abcam). The predicted molecular weight of myostatin is 42.76 kDa, however, this immature myostatin undergoes two cleavage events; one to remove the signal peptide and a second cleavage event to split the protein into two N- and C-terminal fragments. The predicted weights of these fragments are approximately 30 and 13 kDa respectively. The commercial antibody used here was capable of detecting an immunoreactive protein migrating at approximately 43 kDa which is close to the size of the full length precursor monomer (ie including the signal, N-terminus and C-terminus). Quantification of this 43 kDa protein did not show any significant difference in protein expression between the three time points analysed (baseline, 4 h post- and 24 h post plyometric jump protocol).

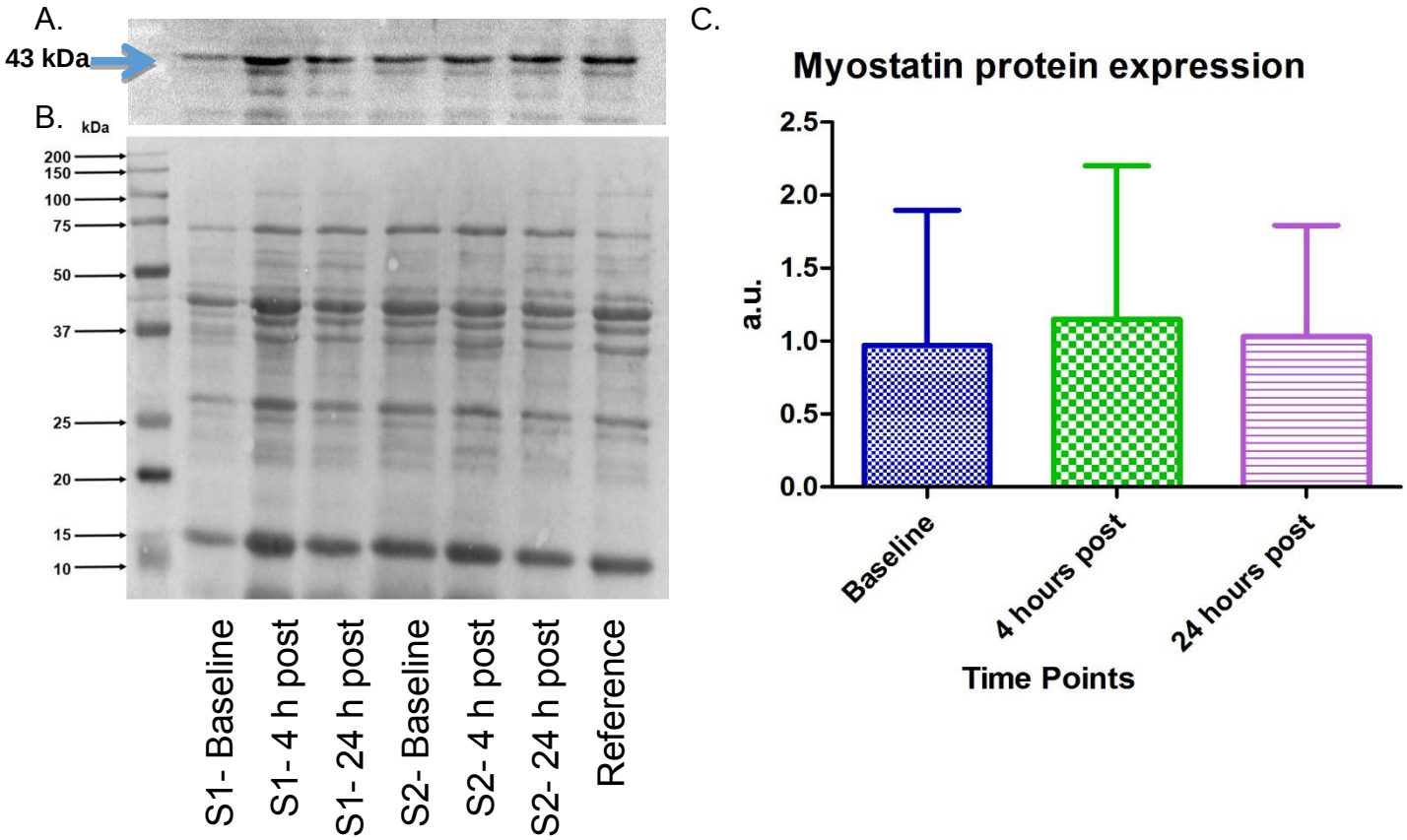


Figure 3.3 Myostatin protein analysis – A) Myostatin precursor protein visualized and indicated at 43 kDa.**B)** Successful transfer of proteins onto the membrane and visualized by ponceau stain, serving as normalization for protein loading**. C)** No significant changes were observed in relative protein concentration over time in response to eccentric exercise. (n=10) S= Subject (Reference sample was an human skeletal muscle sample used at the same concentration across all blots)

3.4.2 Activin IIb receptor

As discussed previously (175,181) myostatin exerts it cellular effects via binding to the activin receptor complex. Therefore it was of interest to determine whether protein expression levels of the activin receptor were altered in response to plyometric exercise training. A commercial antibody which was raised against a recombinant human fragment immunogen was used (Anti-Activin Receptor Type IIB antibody, ab10596, Abcam). The predicted molecular weight of the activin receptor is 57.7 kDa. An immunoreactive protein was detected which migrated at 55-60 kDa (top band on Figure 3.4.A). A faint second band was detected at approximately 50 kDa. The identity of this second band is currently unknown. Quantification of the approximate 55-60 kDa immunoreactive protein found no significant differences in protein expression between time points.

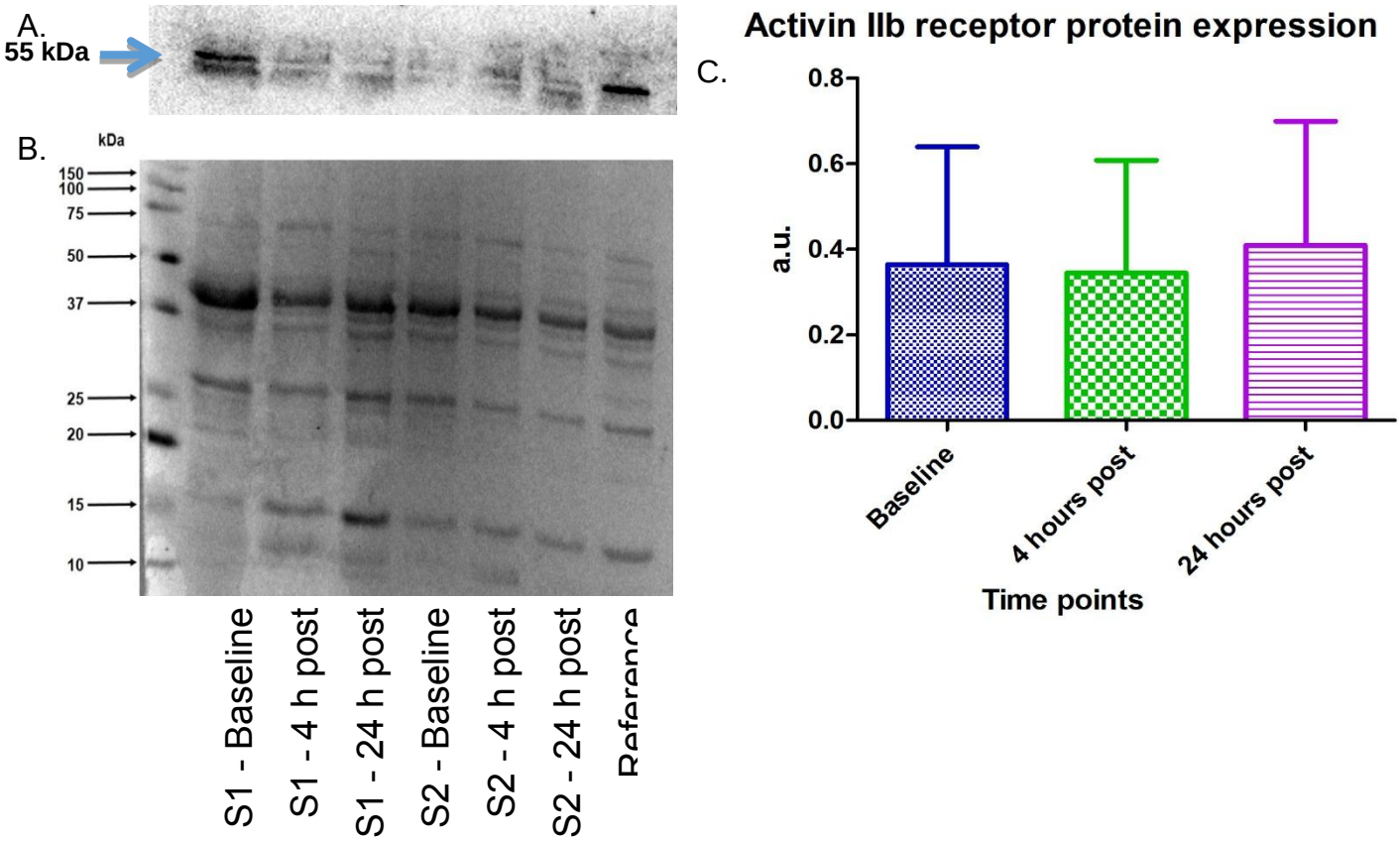


Figure 3.4 ActivinIIb receptor protein analysis - A) ActivinRIIb protein observed at 55 kDa.**B)** Successful transfer of proteins onto the membrane and visualized by ponceau stain, serving as normalization for protein loading.**C)** No significant differences in protein concentration across time (n=7) S= subject (Reference sample was an human skeletal muscle sample used at the same concentration across all blots)

3.4.3 Phosphorylated Smad 2 and 3

Post myostatin binding to the activin IIB receptor an intracellular signalling cascade is initiated whereby the Smad proteins play a key role in transmitting the anti-muscle growth properties of myostatin (discussed in detail in Zhu et al (2004)(185)). Measurement of phosphorylated Smad (pSmad) 2 and 3 levels was conducted via the use of a commercial antibody (Anti-p-Smad 2/3 (Ser 423/425) antibody, sc-11769, Santa Cruz) which was raised against a short proprietary amino acids sequence containing Ser 423 and Ser 425 and which could detect both phosphorylated Smad2 and 3 by means of a homologous region in both Smad2 and 3. The predicted size of Smad2 and 3 was 52.3 kDa and 48 kDa, respectively. A quite faint immunoreactive protein was detected at approximately 50 kDa, which is in close size agreement with pSmad2 and 3. No second immunoreactive protein was clearly detected therefore the immunoreactive protein that was detected at approximately 50 kDa was assumed to represent both pSmad2 and 3.

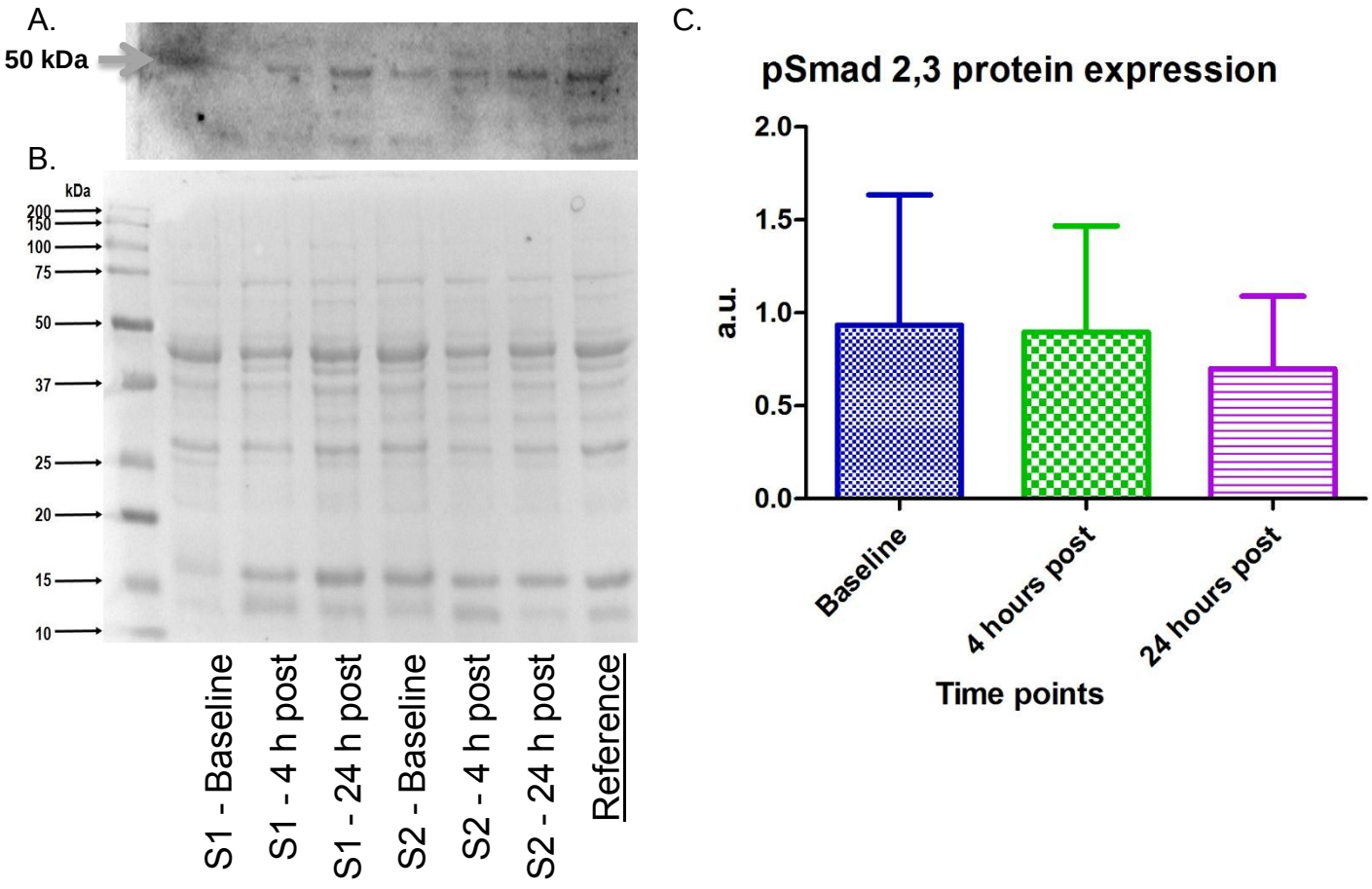


Figure 3.5 Phosphorylated Smad 2,3 protein analysis –A) pSmad2,3 proteins visualized and indicated at 50 Da. **B)** Successful transfer of proteins onto the membrane and visualized by ponceau stain, serving as normalization for protein loading**. C)** No significant changes were observed in relative protein concentration over time in response to eccentric exercise (n=9) (Reference sample was an human skeletal muscle sample used at the same concentration across all blots)

3.4.4 Smad 7

While the Smad proteins 2 and 3 are positive regulators of myostatin signalling Smad7 is a negative regulator. Therefore it was of interest to investigate Smad7 protein levels to ascertain whether there were increased or decreased Smad7 levels in response to the plyometric jumping protocol. A commercial antibody directed towards the N-terminal region of Smad7 was utilised (Anti-Smad6/7 antibody (N-19), sc-7004, Santa Cruz). Within the NCBI database 3 isoforms of human Smad7 as listed with molecular weights of 46.4, 23.9 and 46.3 kDa. The commercial antibody used here detected immunoreactive proteins migrating at approximately 50-55 kDa (Figure 3.6 A). The lower immunoreactive protein that migrated at approximately 50 kDa and hence closer in size to the predicted 46.4 kDa Smad7 protein was quantified. No significant difference was observed in expression levels between the time points (see Figure 3.6 C).

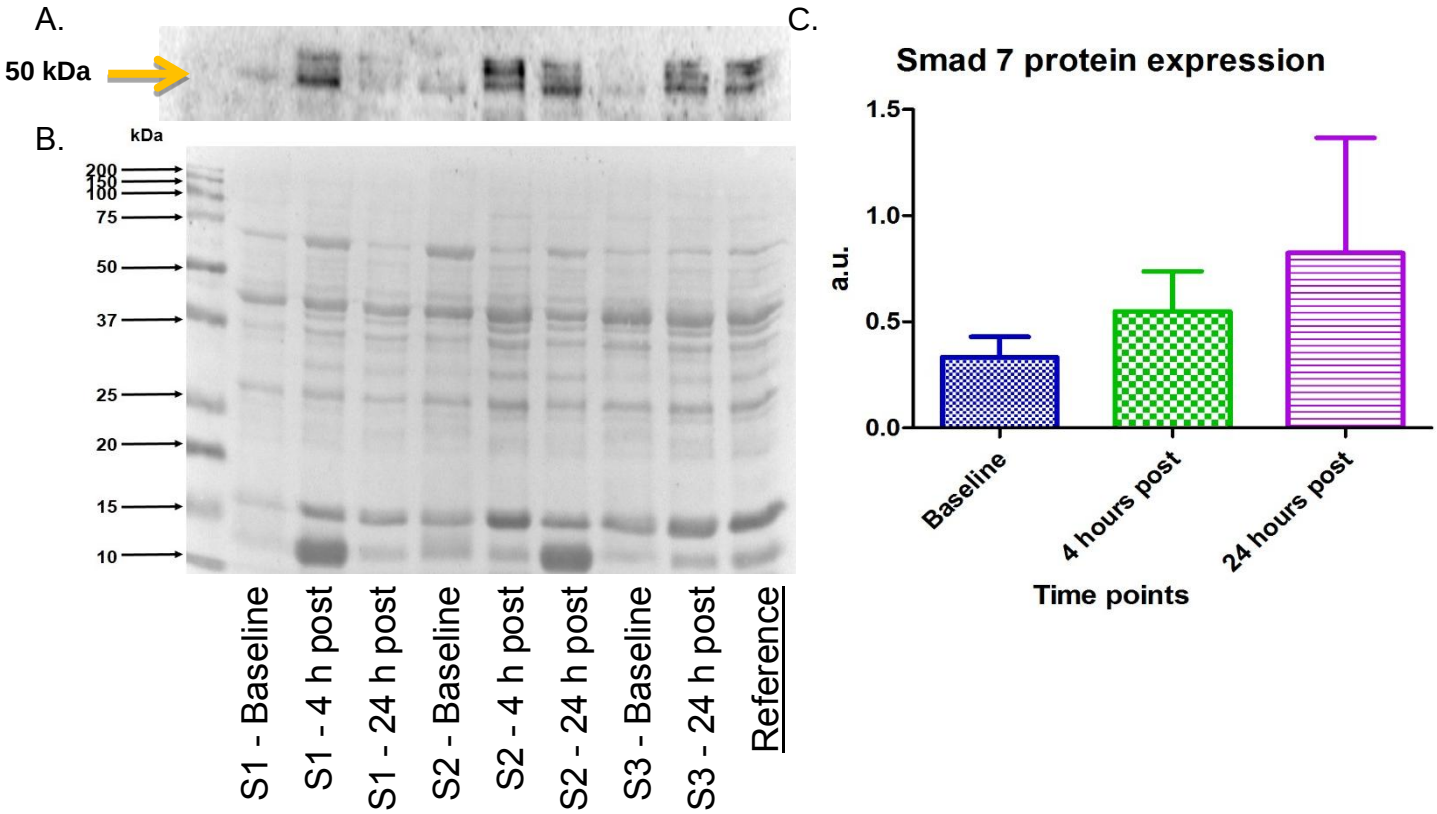


Figure 3.6 Smad 7 protein analysis – A) Smad 7 protein visualized and indicated at approximately 50 kDa**. B)** Successful transfer of proteins onto the membrane and visualized by ponceau stain, serving as normalization for protein loading**. C)** No significant changes were observed in relative protein concentration over time in response to eccentric exercise (n=10) (Reference sample was an human skeletal muscle sample used at the same concentration across all blots)\

3.4.5 Follistatin

Follistatin is a negative regulator of myostatin signalling as it can bind to and subsequently inhibit receptor and downstream signalling activation (189). To assess for follistatin protein expression a commercial antibody (Anti-follistatin antibody, ab47941, Abcam) was utilised which was raised against a recombinant full length follistatin. The predicted molecular weight of follistatin is 38 kDa and an immunoreactive protein migration at approximately 38 kDa (Figure 3.7 A) was observed and is in agreement with the size reported in other literature (239). Follistatin protein expression was significantly increased at 4 h (2.1 fold increase of the means, p = 0.007) and 24 h (2.43 fold increase of the mean p = 0.004) post plyo compared to baseline levels (p < 0.01)(Figure 3.7 C).

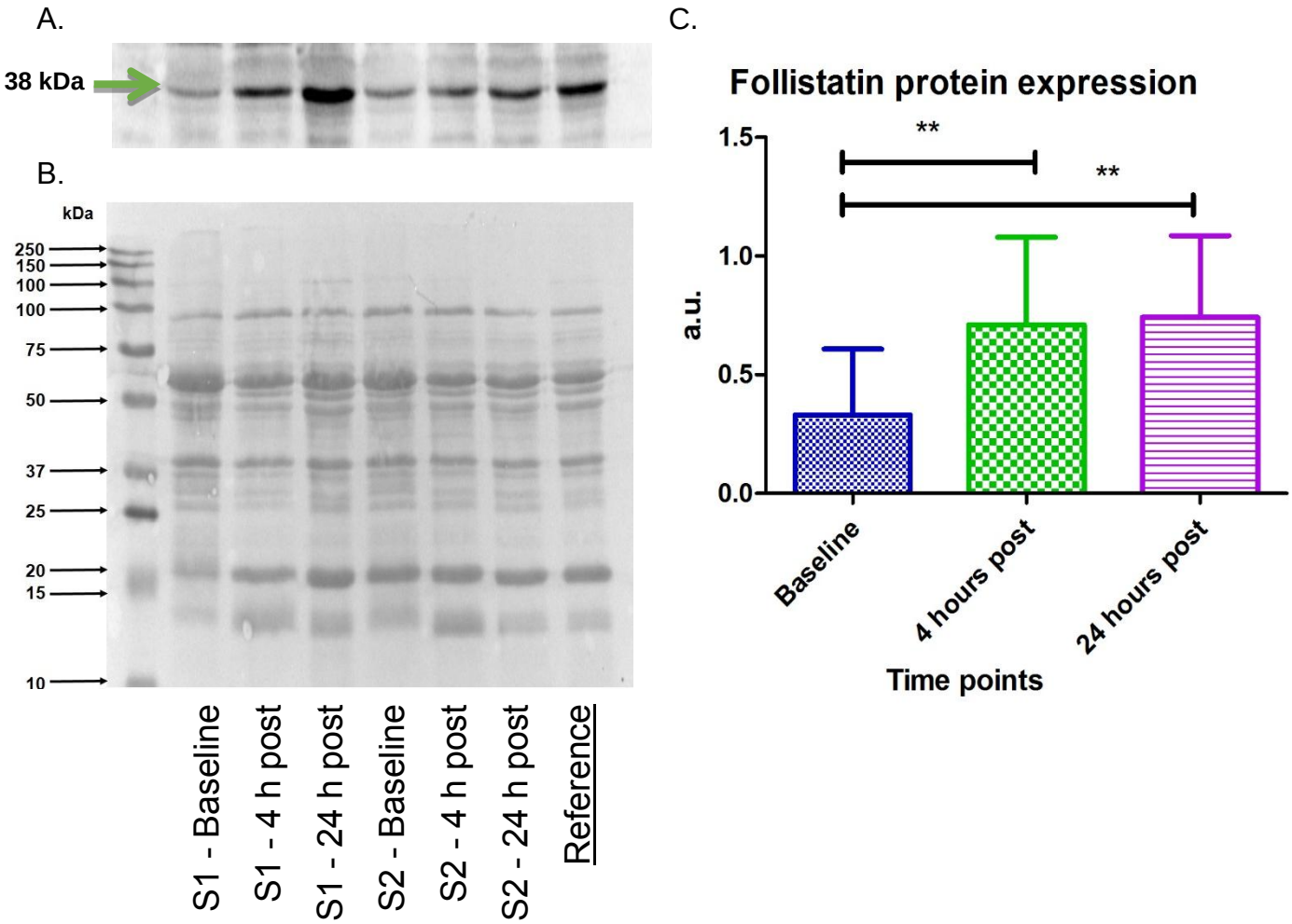


Figure 3.7 Follistatin protein analysis – A) Representative follistatin Western blot image with detection of immunoreactive protein migrating at 38kDa **B)** Successful transfer of proteins onto the membrane and visualized by ponceau stain, serving as normalization for protein loading **C)** Significant changes were observed in relative protein concentration between baseline and 4 h post exercise and also between baseline and 24 h post exercise (p< 0.01)(n=12) (Reference sample was an human skeletal muscle sample used at the same concentration across all blots)

3.4.6 Decorin

Decorin mRNA

Decorin has been reported to be capable of binding myostatin (191) and hence it may also exert regulatory control of myostatin. Currently there is limited or no expression information relating to decorin in adult human skeletal muscle. Therefore the NCBI gene database (http://www.ncbi.nlm.nih.gov) was investigated to obtain information relating to the human decorin gene. Multiple splice variants of decorin were found to be present in the human genome as shown in Figure 3.8

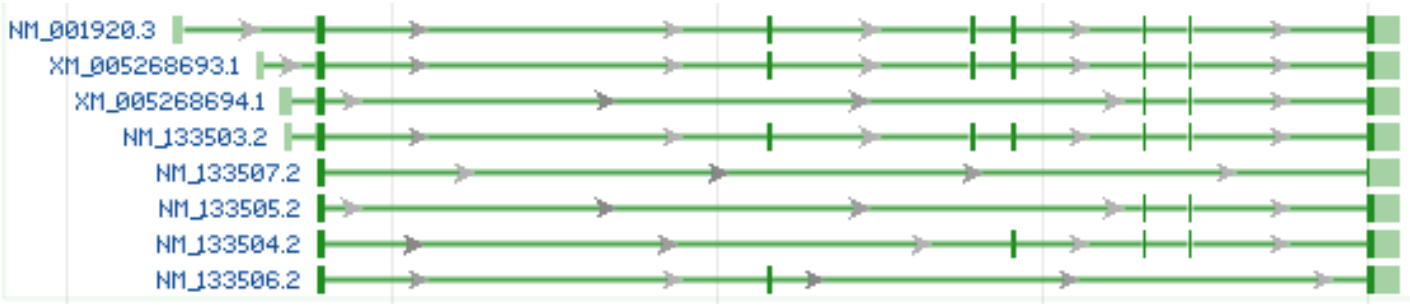


Figure 3.8 Decorin mRNA variants and exon map [Adapted from http://www.ncbi.nlm.nih.gov/gene/1634]

Due to financial and time restraints a focus of investigation was placed on two decorin mRNA transcripts NM_001920.3 hereafter referred to as A1 and NM_133503.2 hereafter referred to as A2, which both encode the same decorin protein, however their transcription start sites differ. Such a difference is suggestive that they may be under different regulatory control by transcription factors. Primer sets were designed that were specific to each transcript and subsequently PCR was performed on cDNA obtained from 3-5 of the participants at baseline, 4hr and 24hr post plyometric exercise. Primer sets 1 and 2 that were specific to A1 and A2 respectively were both predicted to detect amplicons at a size of 260 nucleotides respectively. In all samples analysed amplicons of the expected size were obtained for A1 and A2 (see Figure 3.9). To confirm unambiguously, the identity of the amplicons they were excised from the agarose gel and sequenced. The sequence data obtained confirmed the amplicons to be the decorin transcripts A1 and A2.To obtain further information relating to decorin mRNA expression quantitative PCR (qPCR) was performed using TaqMan® probes. One probe was designed to pick up both decorin transcripts while a second probe was designed to pick up just the A2 decorin transcript. At the time of conducting these experiments no commercially available TaqMan® probe was available to specifically detect decorin transcript A1.

No significant difference was found to be present in mRNA expression levels of decorin when either both decorin transcripts A1 and A2 were measured or when only decoding transcript A2 was measured (see Figure 3.10 A and B).

Decorin Protein

To investigate decorin protein expression a commercial antibody that was directed towards aa 1-359 of decorin was initially utilised (Anti-decorin antibody, ab54728, Abcam). However no signal was detected (data not shown). The decorin protein is highly post-translationally modified (209) and hence these modifications may affect antibody binding. Therefore an alternative strategy was employed whereby decorin was treated with ABC chondroitinase an enzyme that catalyzes the eliminative degradation of polysaccharides containing (1-4)-β-D-hexosaminyl and (1-3)-β-D-glucuronosyl or (1-3)-α-L-iduronosyl linkages to disaccharides containing 4-deoxy-β-D-gluc-4-enuronosyl. ABC chondroitinase is regularly used in studies pertaining to decorin (240–242) and a study by Li et al. (2013)(243) illustrated the differences in immunoblotting results obtained from samples treated with the enzyme compared to untreated. Results from SDS PAGE and immune detection found two immunoreactive proteins migration at 42 kDa and 38 kDa which are likely decorin proteins as similar sized proteins have been reported by Li et al. (2013)(243).However additional antibodies will be required to be used to unambiguously confirm these immunoreactive proteins to be decorin and also to give clearer bands which can be accurately quantified. Such experiments are planned for the future however they were outside the time scale available to this project and hence no quantification of decorin protein expression levels was performed.

3.4.6.1 Decorin - Semi-quantitative PCR

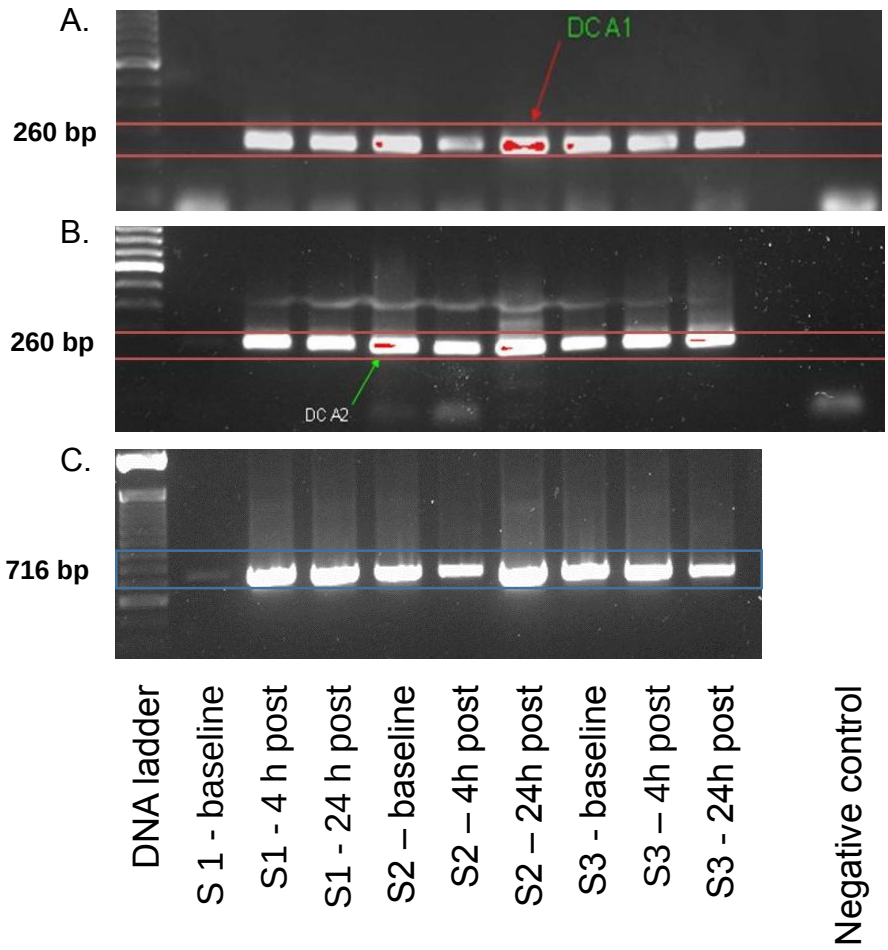


Figure 3.9 Semi-quantitative mRNA analysis of decorin splice variants – A) Agarose gel image obtained with Decorin primer set 1 specific for decorin transcript A1**.B)** Agarose gel image obtained with Decorin primer set that was specific for decorin transcript A2 **C)** GAPDH primer set that served as positive control for quality of cDNA. S= subject (Negative control possessed all the ingredients for the reaction, except DNA substrate).

3.4.6.2 Decorin – Real time PCR (RT-PCR)

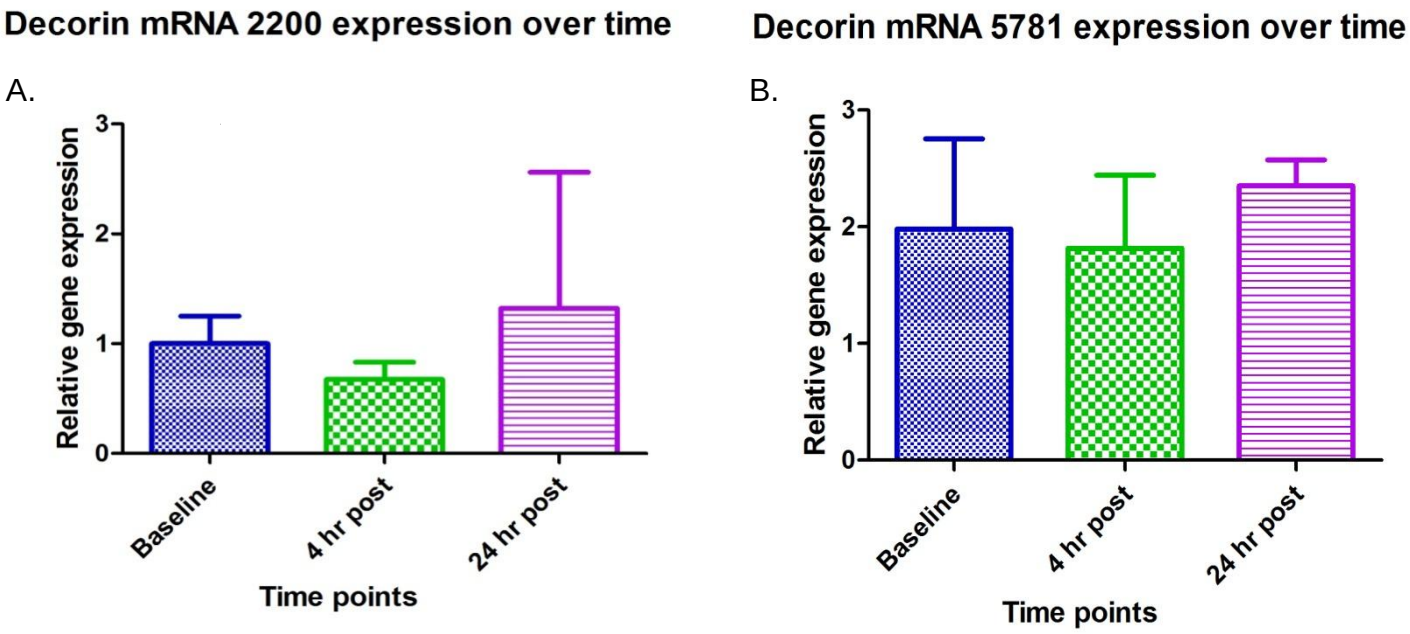


Figure 3.10 Quantitative analysis of decorin variants – A) Decorin gene expression over time for probe 2200: Analysis on the expression of Decorin with the designated probe, revealed no significant differences (n=4). However, the graph illustrated a slight pattern in which expression is lower at 4 hours post exercise compared to the other time points**. B)** Decorin gene expression overt time for probe 5781: Expression of decorin A1 and A2, over the selected times reveal no significant differences (n=5), but it can observe that higher levels of decorin expression are present at 24 hours post exercise.

3.4.6.3 Decorin – protein

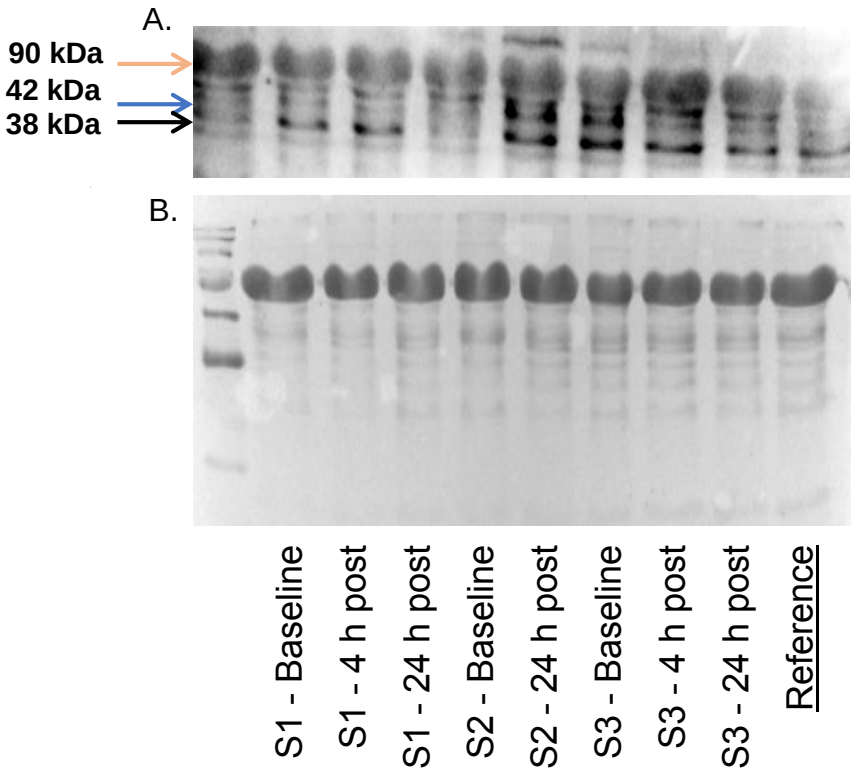


Figure 3.11 Decorin Western blot analysis after enzyme treatment - A) The blot shows bands corresponding to the size of the chondroitinase ABC enzyme (90 kDa) used to cleave the glycosylated side chains, whereas the decorin protein band is shown close to the 42 kDa. Additional band at 38 kDa may be isoforms not yet analysed. The ponceau S image **(Figure B)** showed successful transfer of proteins onto the nitrocellulose membrane, but also revealed changes in the appearance of the other proteins on the blot when compared to the images obtained from other Western blot analysis, which do not use the enzyme (data not shown*). Note – subject 2 (S2) only has two time points biopsies, because of a logistical error during the study*

4 Chapter 4: Discussion

4.1 Model: an acute bout of unaccustomed eccentric exercise induces muscle damage

4.1.1 Creatine kinase

The eccentric exercise employed in this thesis was plyometric jumping. When performing plyometric jumps the knee extensors muscle groups are forced to undergo eccentric contractions and it is from this muscle group whereby biopsies were obtained. Previously this mode of exercise has been reported to induce muscle damage via disruption to the sarcomere structures (28). The indirect skeletal muscle damage indicator creatine kinase was used here in order to confirm the occurrence of muscle damage while maximal isometric muscle contractions were measured to assess for the impact of muscle injury on force output. Post-performance of plyometric jumps creatine kinase levels were significantly elevated at both the 4, 24 and 48 h time points compared to baseline levels. These increases in creatine kinase levels are strongly supportive of the occurrence of skeletal muscle damage as CK is typically bound to the I-band within the sarcomeres and to the M-line of the sarcoplasmic reticulum (47) and hence is intracellular and not in the serum. Performance of eccentric muscle contractions is known to generate increased tension within sarcomeres (26). The increased tension leads to disrupted sarcomeres (23,29) and also ruptured membranes thus likely impacting on the anchors that typically keep CK intracellular. Loss of CK anchorage and membrane rupture would result in CK release into circulatory system. CK levels peaked 24 h after the plyometric jumping and had decreased from these peak levels at 48 h post exercise.

A similar pattern of CK serum levels has previously been reported in a study performed by our research group using the same eccentric (plyometric jumping) exercise protocol (28). The referred study reported values of 529 ± 318 SD U/L at 1 day post exercise versus 627 U/L ± 497 SD in the current study. We emphasize this study by Macaluso et al. (28) as not many studies exist which use the plyometric jumping as the form of eccentric exercise and for the fact that damage was confirmed by several methods including serum creatine kinase, immunofluorescence analysis of membrane disruption and transmission electron microscopy (TEM) analysis of Z-disk streaming. The analyses revealed loss of dystrophin staining showing that type II fibers were more susceptible to damage from plyometric jumps, while TEM showed structural disorganization in post exercise samples (28). Due to unforeseen circumstances, no immunohistochemistry (H & E staining) or immunofluorescence (dystrophin staining) could be performed in the present study, which may have revealed more about the extent of damage sustained during the exercise intervention.

However, the use of serum creatine kinase analysis in this study was mainly to report significant data indicating that skeletal muscle damage did indeed occur.

The significant increase in CK at the 24 h time point coincides with the time point at which the peak presence of neutrophils is observed in injured muscle (59). The high concentration of enzymes released by activated neutrophils in skeletal muscle at this time point likely represents secondary damage occurring as a result of mechanisms for the removal of disrupted myofilaments and other cytosolic structures (244), hence resulting in increased CK release. The reduction in CK levels at 48 h compared to 24 h likely represents a combination of creatine kinase clearance by the kidneys, creatine kinase degradation in the circulatory system and also of reduced creatine kinase release from skeletal muscle.

4.1.2 Reduced isometric force production

Performance of eccentric exercise has previously been reported to result in decreased force production capabilities of the exercised muscle (67). Results obtained during the course of this thesis are in agreement with such findings. The knee extensors muscle group of participants which is a muscle group that is subjected to eccentric contractions during plyometric jumping showed a significant decrease in maximal isometric force production immediately post performance of the plyometric jumping exercise. This reduced force output is likely largely explained due to the structural damage that has previously been reported to occur in the sarcomeres of eccentrically contracted muscle during unaccustomed eccentric exercise (32). Interestingly the attenuated force production capabilities reported here in this thesis (10% reduction after exercise) do not concur with other studies that report larger decreases in force production post eccentric exercise (32-50%, depending on the angle)(64,67–69). Furthermore we observed that 1 week post performance of the plyometric jumping exercise that there was no significant difference in force output levels compared to baseline levels which is in contrast to previous studies which have reported decreased muscle force outputs lasting for several weeks following performance of unaccustomed eccentric exercise (70).

The reason for these contrasting results may be due to different modes of eccentric exercise used and hence, depending on the mode of exercise (e.g. plyometric jumping, eccentric barbell squats (67) or isokinetic dynometer (64), more or less damage may be accrued by participants. An analysis of sarcomere disruption in response to different eccentric modes of exercise would be useful in ascertaining which modes of eccentric exercise induce the least or greatest sarcomere disruption. The use of a uniquely built apparatus for our laboratory may also attribute to differences in results when comparing to other apparatus. Due to relatively large differences observed in force reductions between this study and those mentioned previously, it is likely a combination of factors that are responsible for the differences. Further studies using this apparatus, may need to use the

same exercise protocol as used in this study, to conclude if the variance, when comparing to other studies results, are caused by the apparatus or from inexperience occurring in this study.

In combination the elevated CK levels and reduction in maximal isometric force production provide support that the plyometric jumping protocol used here exerted a stress upon skeletal muscle that should have stimulated hypertrophy signalling cascade. Therefore a suitable environment was created for the investigation of myostatin and some of its associated regulatory factors in order to gain insight into the temporal signalling process that occurs to enable hypertrophy of skeletal muscle in response to eccentric contractions (227).

4.2 Human skeletal muscle myostatin response to eccentric exercise

Removal of myostatin from the mouse and human genome results in the production of significantly more muscular mice and humans respectively (142,147). While elimination of genes is certainly a useful technique in identifying their physiological function it does not provide any information on how the “knocked out” gene achieves that function. Therefore considering that the vast majority of the human population have a functioning myostatin gene present in their genome it is important to learn more about how it functions in promoting skeletal muscle growth so that these pathways can then potentially be targeted to improve muscle hypertrophy when required. Investigation of myostatin protein expression in the acute stages post performance of the plyometric jumping protocol did not show any significant difference in expression levels of an approximate 43 kDa protein that closely matches the predicted size of full length myostatin. This seems to be in line with some exercise studies (206,207,230), while it may be somewhat unexpected when considering that most studies reported a decrease in myostatin mRNA after exercise (see Table 1.1)(228,232,233,235).

It is interesting to note that the full length or precursor myostatin undergoes two separate cleavage events in order for it to be able to bind to the activin type IIb receptor and hence become biologically active (176). Fragments of different sizes 26-30 kDa (mature dimer) and 13 kDa should therefore be detectable. However, none of these fragments were clearly detectable with the commercial antibody used here which was directed towards the C-terminal region of myostatin. Such a finding is suggestive that very limited myostatin cleavage typically occurs in human muscle at baseline levels and during the acute phase following plyometric jumping thus making detection of the biologically active C-terminal portion of myostatin difficult. Also, additional antibodies may need to be used which have a higher affinity levels than the one used here which may have more success in detecting the C-terminal active dimer. It may be wise to use many different myostatin antibodies, to clearly detect all different myostatin forms which, along with genetic and protein analysis of the enzymes responsible in the post expression processing, may clarify this whole process and how it is affected by exercise compared to maybe an atrophy setting like cachexia.

The clear detection of the 43 kDa myostatin protein without the clear detection of the mature active myostatin protein, demonstrates that the majority of myostatin present in adult human skeletal muscle is in its inactive forms at baseline and at the time points after exercise. At these time points the volunteers were in the rested state, thus highlighting the importance of also assessing the myostatin regulatory factors which may have longer half-lives than active myostatin. It will therefore be of future interest to examine some of the proteins that have been reported to be capable of cleaving the myostatin precursor protein and producing the biologically active form. Overall the findings presented here shed new light on myostatin protein expression in adult human muscle as most studies to date have investigated myostatin changes by assessing expression only at the mRNA level in humans (229,230,232,235,245).

4.3 Downstream signalling of myostatin in response to eccentric exercise

4.3.1 Activin type IIB receptor

For myostatin to exert its effect on muscle cells it must bind to its receptor activin type IIb receptor (175). No data is currently available regarding the activin type IIb receptor in human skeletal muscle post performance of eccentric exercise such as plyometric jumping (according to Pubmed searches). Analysis of its expression with a commercial antibody revealed the presence of two closely sized immunoreactive proteins that migrated at approximately 55 kDa and 60 kDa. The reason for the detection of two bands is possibly that it is a result of post translational modifications. The use of second antibody may clarify the legitimacy of this suggestion. Alternatively, non-specific binding of the primary antibody or simply prosthetic groups may explain the second band. The bigger of these two proteins more closely matched the predicted size of receptor (58 kDa) and displayed a more consistent and detectable expression pattern. Quantitative analysis did not yield any specific differences in expression however significant variation was observed among subjects. With variations in expression of approximately 5-6 fold. This variation requires further investigation in a larger group of people to establish whether receptor expression levels may be linked to smaller muscle mass as it could be hypothesised that more receptors may result in more efficacious myostatin signalling. In partial agreement with such a hypothesis it has been reported that treatment of human skeletal muscle cells *in vitro* with an antibody that targets activin type II receptors (and hence prevents myostatin binding) induces hypertrophy (175). Additional information on the expression pattern and cellular localisation of the activin type IIb receptor in human skeletal muscle post eccentric exercise and at later time points during the recovery period will provide useful information on how this receptor may function in regulating muscle hypertrophy *in vivo*.

4.3.2 Phosphorylated Smad 2 and 3

Downstream of activin type IIb receptor in the myostatin signalling pathway lies the Smad family members 2 and 3 that are phosphorylated in response to myostatin binding (184,185). In agreement with our data showing no change in myostatin protein levels no significant change was observed in pSmad2 and 3 levels. This suggests that during the acute stages (24hr) of recovery following the plyometric jumping protocol, there is no involvement of the myostatin signalling pathway and that it is likely at later time points whereby myostatin may exert its effect on skeletal muscle hypertrophy. Further support for such a hypothesis are the results that were obtained for Smad7 which is known as the inhibitory Smad due to its ability to inhibit the Smad signalling pathway via the interruption of Smad phosphorylation at the Activin receptor intracellular kinase domain (187). Smad7 expression levels have been reported to be controlled by the Smad3/4 complex as this complex binds within the promoter region within Smad7 (246). The Smad3/4 complex is formed only when Smad3 is phosphorylated (185) such as that which occurs in response to myostatin binding to the activin type IIb receptor (184).

This regulation of Smad7 by the Smad3/4 complex therefore creates an autoregulatory loop for myostatin signalling and an additional read out of its activity which as mentioned previously, appears to be very low during the acute stages following plyometric exercise. It will be of future interest to study later time points post performance of eccentric exercise to gain a better understanding of the temporal expression of the Smad proteins 2/3 and 7.

4.4 Myostatin regulatory proteins

4.4.1 Follistatin

One of the proteins in the human proteome that is capable of regulating myostatin function is follistatin, which is an autocrine glycoprotein. Follistatin can bind to myostatin and hence inhibit myostatin's ability to interact with the activin IIb receptor (175). Thus follistatin was a protein of interest for this thesis. Present within the human proteome are two known isoforms of follistatin termed Fst317 (317 aa, NP_006341.1) and Fst344 (344 aa, NP_037541.1). No study to date has specifically investigated if both isoforms are present in human skeletal muscle. Results from Western blots performed on protein lysates of participants highlighted the presence of an approximate 38 kDa sized protein that closely matches the predicted size of Fst344 and also matches the predicted size as per antibody datasheet. No clearly detectable immunoreactive protein at approximately 34.8 kDa, the predicted size of Fst317, was present. The commercial antibody used here was raised against a full length recombinant follistatin. Therefore the specific antigen recognition site/s of the antibody is unknown thus this antibody may not be able to detect Fst317. Alternatively Fst317 may not be expressed in adult human skeletal muscle. An analysis of

follistatin mRNA in human skeletal muscle should be performed to rule out the presence of Fst317. It is noteworthy that when the Fst344 isoform is virally expressed in macaque monkeys that significant muscle hypertrophy occurs (247). In light of such a finding it is interesting that the approximate 38 kDa protein detected here, that matches the predicted size of Fst344, was significantly upregulated at both 4 and 24 h post completion of the plyometric jumping exercise. This increase in follistatin is likely to create a favourable environment for muscle regeneration and growth. How follistatin achieves the promotion of skeletal muscle hypertrophy is still unclear. One potential mechanism is through aforementioned negative regulation of myostatin. However when follistatin is overexpressed in mice from a myostatin null background significantly more muscular mice develop compared to mice that are only myostatin-/- (203). Such a finding provides support that follistatin interacts with other possible negative muscle regulators or promotes the activity of positive muscle growth regulators. With developments in whole proteome analysis such as the use of Mass spectrometry (248) it will be of interest to explore the skeletal muscle proteome of mice who overexpress follistatin and identify the proteins that are up and down regulated in this model. Results from such experiments should provide additional targets to investigate in humans that may be involved in regulating muscle hypertrophy.

The increased follistatin protein expression reported here is in contrast to a report of a study that investigated follistatin mRNA expression in the *vastus lateralis* of young males. This study reported no difference in expression after performing an eccentric exercise protocol consisting of 6 sets of 12-16 maximal single leg knee extensions on an isokinetic dynamometer at 60º/s (249). In the latter study, muscle biopsies were taken 11-15 days before the first exercise session and 24 h after the completion of the single bout of exercise. An additional study also investigated follistatin mRNA expression post performance of mainly sub-maximal (55%-75% 1-RM) concentric movements, consisting of both upper body (chest press, shoulder press and lateral pull down) and lower limbs movements (leg press variations). The results showed no significant difference in expression levels in muscle biopsies taken 9 h after post-exercise overnight recovery (250). These findings highlight the need to examine both mRNA and protein levels in order to obtain a more complete picture of how a gene functions in a physiological context.

The upregulation of follistatin protein levels may be at least in part due to testosterone since the treatment of primary murine skeletal muscle cells with 100 nM of testosterone resulted in an increase in follistatin protein levels (251). Testosterone levels are increased as early as 15 min after exercise in humans consisting of either concentric or eccentric contractions performed during four different exercises: bench press, leg curls, leg extensions and military presses. During the eccentric exercise trial, the bars or machines were lifted and the subjects controlled the lowering of the weight, negative part of the motion (252). The study by Durand et al. (2013) therefore fit within the time frame for the increased follistatin protein expression at 4- and 24 h post plyometric

exercise. To assess for the possible role of testosterone in follistatin regulation after eccentric exercise, subjects who have low testosterone levels could be compared to those who have high testosterone levels. Furthermore, exercise studies could also be done to simply assess the correlation between testosterone and follistatin, which may suggest a connection between the two factors. If this was the case, it could stimulate more cell studies to reveal the mechanism for this association.

The increase of IGF-1, and its isoforms (253,254) after exercise may also indirectly contribute to the increase in follistatin. This suggestion is based on information from studies on quail and duck, reporting that IGF-1 significantly increased the follistatin mRNA levels (209,210). When considering these reports, it could be suggested that the plyometric jumping (eccentric muscle contractions) stimulated the increase in IGF-1 which in turn significantly upregulated the expression of follistatin observed at 4 h and 24 h after exercise. However, future studies need to be done that also include analysis of circulating IGF-1 and muscle specific IGF-1, along with their isoforms, to validate our proposed suggestion.

4.5 Decorin variants in human skeletal muscle

Similar to follistatin, decorin has also been reported to be capable of binding myostatin (191) and hence may exert regulatory control over myostatin. There has been however no reported investigation of decorin expression in adult human skeletal muscle. Currently the main role ascribed to decorin in skeletal muscle is in fibrosis (255–257). Our findings therefore, with regards presence of two decorin splice variants termed here A1 and A2 in human skeletal muscle, are particularly noteworthy as presence of both of these transcripts has not been previously reported in the published literature. Both transcripts A1 and A2 are predicted to encode for the same decorin protein however their transcription initiation sites differ and hence suggest the existence of two different promoters for regulating decorin mRNA expression in adult human skeletal muscle *in vivo*.

Such an occurrence likely results in different transcription factors being involved in the regulation of A1 and A2 transcription. Analysis of decorin mRNA expression using Taqman® probes that either detected both transcripts A1 and A2 or solely the A2 transcript did not demonstrate any significant changes in decorin expression at any of the time points analysed. At the time of doing these experiments there was not a commercially available Taqman® probe to measure solely the A1 decorin transcript. It will be of future interest to develop such a probe as it will provide information on whether skeletal muscle preferentially upregulates either of these two decorin mRNA transcripts in response to eccentric training. Should a difference be observed in transcription of A1 and A2 it will then be of great interest to determine what transcription factors are involved in the regulating the differential expression.

4.6 Decorin protein analysis

Similar to the investigation of decorin mRNA in adult human skeletal muscle no published literature was found in relation to decorin protein expression in adult human skeletal muscle. This is somewhat surprising, as decorin overexpression in C2C12 cells results in the creation of cells with greater hypertrophy than non-overexpressing cells (218). Furthermore decorin was also found to reduce the effects of exogenously applied myostatin to C2C12 cells (218) thereby supporting results from a previous study demonstrating an interaction between myostatin and decorin (191).

In the present study decorin expression was initially investigated with a commercial antibody that was raised against a recombinant full length decorin protein however no signal was detected (data not showed). This lack of signal detection is not unusual as no enzyme was used to cleave any GAG chains attached to the core protein, which is the procedure in most studies investigating decorin with immunodetection (240–242). Therefore protein lysates were subsequently treated with the enzyme chondroitinase ABC before blotting. The chondroitinase ABC enzyme is capable of degrading polysaccharides containing (1-4)-β-D-hexosaminyl and (1-3)-β-D-glucuronosyl or (1-3)-α-L-iduronosyl linkages to disaccharides containing 4-deoxy-β-D-gluc-4-enuronosyl groups (Sigma datasheet). Western blot analysis of enzyme treated lysates with a commercial antibody directed towards the complete region of decorin core protein, resulted in the detection of two distinct immunoreactive proteins that migrated at approximately 38 kDa and 42 kDa. These proteins detected are in close size agreement with those detected by 40-42 kDa and with the predicted size of the decorin core protein. Additional antibodies directed towards different regions of decorin should be used to see if the same sized immunoreactive doublet is observed. Additionally for quantification purposes additional antibodies will hopefully give clearer images that can be used for quantification than those obtained here.

Decorin is a widely expressed protein and hence it will also be of interest to determine whether the GAG chains differ between cell types. In the case of decorin, GAG chains are important for the organization of fibrils (258).The presence of GAG chains on decorin create an interesting issue with regards its detection in situations such as immunocytochemistry and immunohistochemistry. During the course of investigations for this thesis it was found that no decorin protein could be detected in protein lysates obtained from adult human skeletal muscle without prior treatment with the ABC chondroitinase enzyme. Hence investigations relating to the cellular localisation of decorin should also take heed of such findings as it may require pre enzyme treatment of tissue sections before staining otherwise an inaccurate profile of decorin cellular localisation may be obtained. The generation of antibodies that are capable of detecting decorin with its GAG chain still intact would be particularly useful in aiding the investigation of decorin in human skeletal muscle.

5 Chapter 5: Conclusion

In conclusion, this study and thesis examined the effect of eccentric exercise, more specific plyometric jumping, on muscle damage and subsequently the response of the negative muscle regulator myostatin. Additionally factors that regulate myostatin's action i.e. follistatin, Smad7 and decorin were also investigated.

We show that the plyometric jumping protocol used in this study was adequate to produce muscle damage, resulting in increased circulating CK concentrations and reduced voluntary force production capabilities. The study also addressed factors affecting muscle regeneration, both positively and negatively. It was shown that this mode of eccentric exercise did not change protein levels of myostatin, it's receptor (Activin IIb) and the status of downstream proteins Smad2/3 during the first 24 h post performance of the plyometric exercise. Furthermore, the concentration of inhibitory Smad7 also remained unchanged at all-time points. However, the protein concentration of follistatin showed significant increases post-eccentric exercise, making it one of the first studies using this protocol and reporting increased follistatin at protein level.

Lastly, this study is the first investigation into human skeletal muscle revealing the existence of decorin splice variants; however no change in expression of these variants were observed in response to an acute bout of eccentric exercise. This is however a novel finding, opening the door to future research into decorin splice variants and their expression patterns in human skeletal muscle.

5.1 Future research

This study was one of the few which investigated factors capable of regulating myostatin, at protein level. However, this complicate matters when doing the research as the investigation of proteins with more affordable methods like immunoblotting, leads to difficulties as to finding correct antibodies which work with human samples and probe for the correct portion on the protein. In the case of proteins with multiple steps of processing, the variety of antibodies seems to be crucial. This calls for more companies to produce antibodies against several immunogens for the use in human muscle sample analysis. Furthermore, it is important for future studies investigating myostatin, to include quantification at several levels, i.e mRNA, precursor protein and mature dimer, combined with downstream activation in terms of Smad signalling activation and/or transcriptional gene targets (like p21).

It has become clear that there are increasing amount of factors able to bind myostatin and thereby regulating its ability to bind to its designated receptor. Lately, more of the ECM proteins are being included in this group of regulators. Questions arise such as why these proteins bind myostatin, or

is it myostatin which binds them, thereby somehow changing their conformation and subsequently regulating their stability? These questions are also important in future research.

Aside from myostatin, investigation into follistatin at a protein level is also highly recommended in all future studies which investigate myostatin and/or muscle regeneration. Follistatin is another protein which is post-translationally processed. This will need to be taken into account in future studies, possibly calling for immunoprecipation analyses to analyse the interaction between follistatin and myostatin in humans, *in vivo*.

Decorin is becoming a very interesting highly important protein and myokine, investigated in several research areas from cancer and now skeletal muscle. The amount of functions attributed to this protein is astonishing and biochemical structural investigation may lead to clarifying which domains or amino acid sequence within the protein is responsible for functions. Cell models have demonstrated the effects of decorin on myotubes which calls for the change of model, maybe introducing over-expression of decorin in mice skeletal muscle. It would be very interesting to see the effect that overexpression of both follistatin and decorin has skeletal muscle in mice. These two factors together have the ability to regulate most, if not all, the factors which may negatively affect muscle growth. Decorin even goes further to control chronic or irregular fibrosis by binding to TGFβ-1. I would include these factors in models investigating muscle dystrophy.

When investigating human skeletal muscle, it would be strongly suggested to continue with our research into the genetic regulation of decorin to further investigate the newly discovered A1 and A2 variants in human skeletal muscle. This calls for analysis into the transcriptional factors involved in their expression, which will subsequently aid in clarifying the stimulus involved in their expression. The investigation into other variants may also be useful, as there are presently several variants listed on the NCBI database. Downstream of the mRNA, the decorin protein is also crucial and we call for the use of 2 or more antibodies in future studies, after treatment with chondroitinase ABC. This will allow the accurate identification and quantification of the 359 aa (40-42 kDa) decorin protein in human skeletal muscle, as the possibility of decorin isoforms or degraded decorin is promising and realistic. The future research for decorin in human skeletal muscle may be challenging, but taking into account its variety of functions and its potential as a muscle mass regulator, it would be almost absurd not to continue with this research pertaining to decorin.

Techniques / Methods

Further suggestions concerning techniques would be that future studies also make use of different methods to clarify several questions raised concerning, but not limited to, the different forms of proteins and possible isoforms. One of the most effective methods for this suggestion would be mass spectrometry (MS). This allows the investigation of several proteins at once, by cutting out a complete lane in the SDS-PAGE gel. Apart from identifying the proteins, the MS could be

connected to analysers which are able to also sequence these proteins. This would aid in characterizing the possible isoforms of the proteins by amino acid sequence and use online database to identify sequences present in other proteins which may clarify the function of these isoforms. It would suggested that MS be used in both serum and muscle samples where possible.

The use of MS with RT-PCR is the future for research; however, the financial implications associated with these techniques may still be too high to introduce to regular studies.

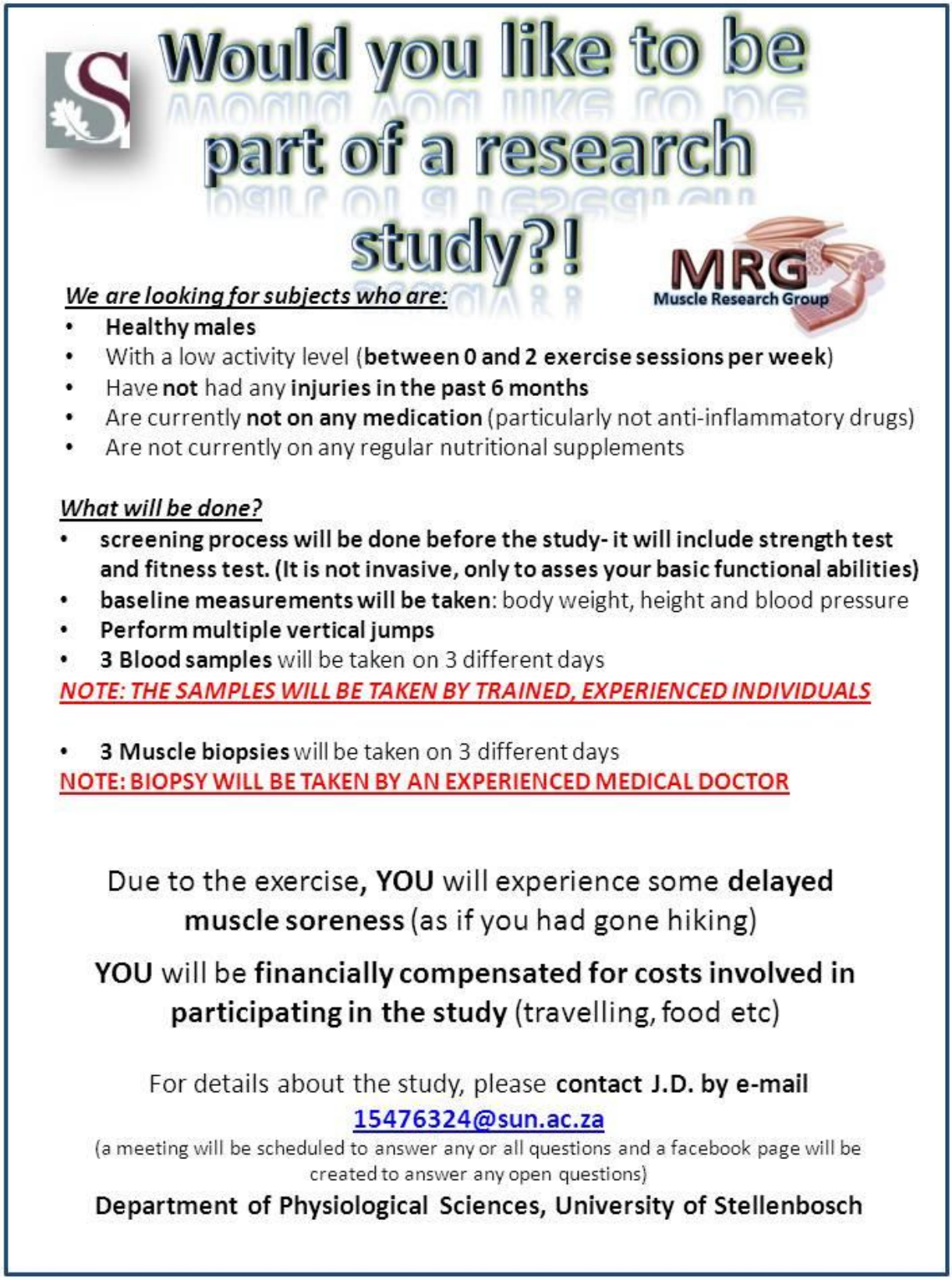
Exercise protocols

In the present study, we used the plyometric protocol which is regularly used within our department. This is effective for the investigation of eccentric exercise on muscle damage and repair, however, all the different research groups seems to have their own protocols. This makes it difficult to compare results between studies. I would therefore suggest that international research group clusters are formed, in which the labs use the same protocol with similar subjects (in terms of characteristics) to evaluate or investigate a factor. By this I mean that if several labs with different specialities are able to work together, it will save time and enable research questions to be answered in full and in a shorter amount of time. In this way it would be able to conclude all different aspects of say plyometric jumping on muscle damage, repair and/or adaptations while looking at a variety of factors at once. This would allow studies to also be compared to each other.

On the topic of exercise protocol, I would strongly suggest a change in regular use protocols to exercise regimes closer to those used by athletes in present times. In the athlete arena, training regimes are changed regularly while science are somewhat lagging behind. This makes it difficult for athletes to use information from scientific studies as this is not always accurate when compared to how they are training.

6 Appendices

Advertisement poster



Informed consent

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

Myostatin: roles in skeletal muscle structure, regulation and regeneration after eccentric exercise

REFERENCE NUMBER:N12/08/051

PRINCIPAL INVESTIGATOR: Prof. K.H. Myburgh

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7600

CONTACT NUMBER: 021 808 3149

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Committee for Human Research at Stellenbosch University and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

This study will be performed at the Department of Physiological Sciences of the University of Stellenbosch.

This study will be focusing on the healing process of skeletal muscle after micro-damage induced by exercise of a type that includes a lengthening contraction. This process is influenced by several factors able to control or affect muscle in a negative or positive way. The muscular influential factors are crucial for determining muscle mass, particularly after micro-damage. Research results don't present a clear picture, but most provided results showing that a factor that limits muscle growth (myostatin) is increased at the beginning of injury, despite the fact that new muscle must be formed.

This study aims to focus on why this myostatin factor plays a role after micro-damage. The study will also be focusing on factors that are studied less than myostatin that are able to bind and reduce the anti-growth effects of myostatin.

The results may 1) indicate if myostatin plays a role in muscle regeneration 2) identify possible factors able to control the effects of myostatin during muscle regeneration.

What will be done?

The study will be conducted in the Exercise physiology laboratory at the Mike de Vries building, (Department of Physiological sciences), Stellenbosch University. We plan to do the actual study using a total of 16 participants (n=16).

Before the start of the actual study, you will be asked to participate in a screening process. This will consist out of a fitness test and a strength analysis test. All the tests will be performed in-house. The participants will be divided into 3 groups (A,B,C) of 10 for the screening process. The study will start with a blood draw (baseline) and a week later the fitness tests will start. After the holiday, the first muscle biopsy (baseline) will be taken and followed by 1stround of strength tests. (The strength tests will be one week from each other).

At this time, we will have collected enough information to conclude which participants will be suited for the study. These 16 participants will continue to the exercise protocol and post-exercise muscle biopsies and blood draws.

Fitness test: Cycling VO2 max test - You will be cycling for one minute periods at an increasingly higher resistance until they can no longer pedal. The subject's peak oxygen consumption rate and peak cycling wattage will be measured and recorded. In order to assess oxygen consumption rate, you will be required to perform the test with a mask placed over their mouth and nose. Subjects will be able to easily inhale room air while expired air will be analyzed for oxygen and carbon dioxide content.

Strength test – A quadriceps isometric force test on a leg extension apparatus set at 70°.You will have 3 attempts to produce your maximum force. The average force of the 3 attempts will be used as your result.

You will be expected to come in to the laboratory 9 times on 8 different days

(A detailed calendar time line is attached, please see pages at the end)

During these visits, the following will happen:

Visit 1 (Blood baseline): First, you will be requested to sign the informed consent form.

Then we will perform *baseline* measurements – weight, height and blood pressure

Baseline blood draw will be collected

Visit 2 (Fitness test): You will perform your fitness, VO2max test

Visit 3 (Muscle Biopsy Baseline): This will be the first visit where a muscle biopsy will be taken from your *vastus lateralis* (upper leg)

Visit 4 (Strength/Force test): First round of strength tests

Visit 5 (Strength/Force test): Second round of your strength tests

Visit 6 (Exercise protocol): *You will be asked to perform the plyometric exercise protocol (Verticalexplosive jumps)*:10-minute warm-up and stretching will be followed by the exercise intervention.

The plyometric regime will consist of 10 sets of 10 repetitions (90% of your maximum height)

Leg circumference and blood draws will be taken prior to jumping

Visit 7 (4 Hours post-exercise): Leg circumference, blood sample and muscle biopsy

Visit 8 (24 hours post-exercise): Leg circumference, blood sample and muscle biopsy (opposite leg)

Visit 9 (48 hours post-exercise): This will be the 5th and final blood draw and also the post-exercise force production test

Plyometric Exercise Regimen:

Prior to the start of the exercise regime, you will be handed a piece of chalk to use as a marker for your three maximal jump attempts. The height of the three chalk marks will be measured from the ground and the average calculated. The height that represents 90% of your maximum jumps height will then be indicated by a strip of tape and this will serve as your target height for each jump. Each jump that does not reach this height, will be counted and you will be asked to perform the additional jumps at the end of the protocol to conclude 100 jumps of 90% of your maximum jump height.

To induce micro-muscle damage, you will be asked to perform ten sets of ten maximal vertical jumps. The sets will be separated with a 60 second recovery period. On landing of each jump, you

will be instructed to adopt a knee joint angle of approximately 90° in order to promote muscle damage as described in scientific literature. This plyometric exercise protocol will induce non-severe muscle damage, which will not reduce your mobility after the protocol is done.

There will be an individual given the task to observe your jumps and to confirm that your finger tips reach your target line.

Muscle biopsies:

Muscle biopsies will be performed by a medical doctor (Dr. J. Brink) using local skin anaesthesia, open surgical biopsies from the thigh muscle will be obtained on different days as described above (*see calendar*). The first muscle biopsy will be performed on a random leg and the following biopsy on the opposite leg, as this will help to reduce any possible differences between the dominant/non-dominant legs. During the procedure a piece of approximately 50-100mg of muscle will be taken. This is a relatively small amount, almost the size of the tip of a ballpoint pen. This procedure will not have a negative influence on your muscle function and your muscle will recover completely within a short period of time. Although, pain may be present at the biopsy site during sports participation for a period of 1-2 days, it is not sufficient to limit sports participation in non-contact sport. You should refrain from sporting activity for 1 day after a biopsy, but are free to recommence participation on day 2 after a biopsy.

Blood collection:

All blood samples will be drawn from the forearm by a qualified phlebotomist (trained and certified to draw blood) using a double sided needle and a standard serum separating tube (SST)

At each time point, 10ml (one teaspoon) of blood will be drawn. A total of 50ml of blood will be drawn throughout the study.

Why have you been invited to participate?

You are invited to participate in this study because your physical characteristics fit with the expected criteria needed for this study and were selected through the strength and fitness screening process.

What will your responsibilities be?

You will be expected to participate in study by doing the activities described above for visits 1-9. Your participation is entirely voluntary and you are free to decline participation or withdraw from the study at any time. If you choose to say "no" or withdraw, this will have no consequences for you, in any way. *Any further questions that you may have relating to the experiment will be answered in full by the co-ordinator: Prof K.H. Myburgh (021 808 3149).

Will you benefit from taking part in this research?

You will have no personal benefits from taking part in this study.

Are there in risks involved in your taking part in this research?

The risks of having blood drawn and muscle biopsies include soreness, bruising at the puncture site and risk of infection. They will be minimized using sterilized equipment and an anesthetic drug only during muscle biopsies. All the procedures will be done by experienced personnel with sufficient relevant training. The amount of blood and muscle to be taken is not considered to be a significant amount, and is therefore not expected to have any significant risk for you.

The risks of having plyometric exercise intervention include delayed muscle soreness and possible low-risk injury. We will however insure that you will warm-up adequately and that you as participants understand the correct form of the exercise to decrease the possibility of injuries. However, should you require medical care as a result of negligence in this study, it will be made available free of charge.

If you experience any pain or discomfort as a result of your participation, please contact the principal investigator, at the number provided at the top of this form.

If you do not agree to take part, what alternatives do you have?

Your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever.

Who will have access to your medical records?

All information will be kept strictly confidential. Experimentation will not commence without your consent. Your identity will remain anonymous. Only Prof K.H. Myburgh and Mr J.D. Conradie will have access to your personal information and to your experimental data. The Research Ethics Committee members, who approved this study, will also have the right to inspect research records.

What will happen in the unlikely event of some form of injury occurring as a direct result of your taking part in this research study?

In the unlikely event that you sustained some form of injury as a result of your participation due to negligence on our side, the University has insurance to cover medical expenses.

Will you be paid to take part in this study and are there any costs involved?

You will be compensated pro rata for your time and any inconvenience for participating in the study, but if you withdraw from the study at any point after you agreed to participate you will only be compensated for the part that you did participate in. There will be no additional costs involved for you, if you do take part. *if you do not proceed past the screening process, we will still compensate you for your troubles up to the point where you participated in the study

Is there anything else that you should know or do?

You can contact Prof K.H Myburgh at tel: 021 808 3149 if you have any further queries or encounter any problems.

You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.

You will receive a copy of this information and consent form for your own records.

The researchers involved in this study have no financial or non-financial interests, which may inappropriately influence us in the conduct of this research study.

Declaration by participant

By signing below, I …….……. agree to take part in a research study entitled *(insert title of study)*.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…….. On (*date*) ……...…….. 2013.

.....	.....
Signature of participant	Signature of witness

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter.

Signed at (*place*) .....…..……. on (*date*) ……...…….. 2013.

....

Signature of investigator Signature of witness

Question form

Principal Investigator: ***Prof. K.H Myburgh***

Sub-investigator: ***J.D Conradie***

Myostatin: roles in skeletal muscle structure, regulation and regeneration after eccentric exercise

Name:	Surname:	US Number:	
Cell no:	Age:	Height:	Weight:

Physical activity and Injury questionnaire:

Physical Activity:

Do you, or did you participating in any competitive sport at university? Y | N

If yes, what sport did/are you participating in?

……

How long ago?

currently: <2 months ago: ☐ 2 months ago: ☐ 6 months ago: ☐ 1 year ago: ☐ > 1 year ago: ☐

Do you currently participate in any recreational physical activity? Y | N

If yes, what? ……..

How often?

< twice a week	twice a week	3 times a week	> 3 times a week
☐	☐	☐	☐

In the past 4 weeks?

< twice a week	twice a week	3 times a week	> 3 times a week
☐	☐	☐	☐

At what intensity?

Low: ☐ moderate: ☐ high: ☐

Do the activities include any eccentric exercises? Y | N

What is your current level of fitness?

Unfit: ☐ Moderately fit: ☐ Highly trained: ☐

Injury history:

Have you had any injury to your lower extremities? Y | N

If yes, where?

Hip: ☐ knee: ☐ Ankle: upper leg: ☐ lower leg: ☐

Of what nature?

Sprain: ☐ Strain: ☐ Contusion: ☐ injury to bone ☐
Dislocation: ☐

How long ago?

< 2 months ago: ☐ 3 months ago: ☐ 6 months ago: ☐ 1 year ago: ☐

> 1 year ago: ☐

Are you currently using any medication or supplementation? Y | N

If yes, please specify …….

Are you currently on any diet restrictions etc? Y | N

If yes, please be specific as to what it attain to ……..

Subject details at the time of the trials

Name, Surname	Code	Age (years)	Height (cm)	Weight (kg)	VO_{2max} (ml/kg/min)
JT Motto-Ros	JTMR	21	179.1	73.6	43.46
Timothy van Niekerk	TvN	20	174.00	70.2	47.9
Julian Wissing	JW	19	179.70	84.2	47
Wesley van der Westhuizen	WvdW	19	181.60	74	50.5
Adarsh Shah	AS	20	178.10	58.4	43.6
WG Klingenberg	WGK	25	177.6	105.2	38.4
Pieter-Simon Basson	PSB	23	185.1	73.2	42.14
Cloete du Preez	CdP	22	180	84.6	43.62
Matthew Bergsteedt	MB	20	173.4	68.8	42.6
Ruan Aucamp	RA	19	181.4	84.2	45.3
Charl Engels	CE	28	186	113.2	38.16
MP Austin	MPA	20	178.5	73.6	55.7
Bradwill Markgraaf	BM	20	178	55	45.08
Tope Ogundipe	TO	20	177	73	44.4
Claus van Wyk	CvW	19	178	63.8	44.1

Individual creatine kinase values (u/L)

Name, Surname	Code	Baseline 1	Baseline 2	4 hours Post	24 hours Post	48 hours post
JT Motto-Ros	JTMR	151	177	164	2390	4980
Timothy van Niekerk	TvN	125	87	106	120	135
Julian Wissing	JW	269	244	256.5	319	618
Wesley van der Westhuizen	WvdW	200	238	219	257	389
Adarsh Shah	AS	162	309	235.5	352	432
WG Klingenberg	WGK	142	115	128.5	180	373
Pieter-Simon Basson	PSB	172	143	157.5	230	518
Cloete du Preez	CdP	123	192	157.5	564	709
Matthew Bergsteedt	MB	100	92	96	543	768
Ruan Aucamp	RA	231	191	211	1776	2167
Charl Engels	CE	120	128	124	351	701
MP Austin	MPA	310	187	248.5	223	346
Bradwill Markgraaf	BM	134	145	139.5	161	370
Tope Ogundipe	TO	455	634	544.5	625	703
Claus van Wyk	CvW	95	106	100.5	419	624

Individual isometric force production values (N/kg)

Name, Surname	Baseline 1	Baseline 2	Highest baseline	Immediately Post	1 week post
JT Motto-Ros	15.24	16.223	16.223	10.901	15.652
Timothy van Niekerk	8.48	8.476	8.48	12.422	12.607
Julian Wissing	11.75	16.591	16.591	13.480	15.499
Wesley van der Westhuizen	11.98	14.311	14.311	13.446	15.162
Adarsh Shah	10.94	14.207	14.207	9.298	10.548
WG Klingenberg	10.97	10.124	10.97	9.905	10.371
Pieter-Simon Basson	12.87	11.503	12.87	10.656	10.888
Cloete du Preez	11.67	12.621	12.621	11.749	11.678
Matthew Bergsteedt	7.94	9.172	9.172	9.724	11.003
Ruan Aucamp	11.64	12.553	12.553	13.005	15.724
Charl Engels	11.79	11.446	11.79	11.696	14.055
MP Austin	11.43	16.386	16.386	14.348	15.285
Bradwill Markgraaf	14.22	14.285	14.285	14.764	14.655
Tope Ogundipe	10.65	11.082	11.082	11.247	11.178
Claus van Wyk	14.11	14.592	14.592	13.683	16.332

RNA analysis – detailed information

Code	Time point	RNA concentration	Decorin 2200 expression	Decorin 5781 expression
BM	Baseline		1.061	3.317
	4 hours post		0.597	1.495
	24 hours post		0.853	2.585
WGK	Baseline		0.886	1.597
	4 hours post		0.880	0.963
	24 hours post		0.570	2.049
CE	Baseline		Outlier values	1.945
	4 hours post			2.639
	24 hours post			2.540
CdP	Baseline		0.740	1.380
	4 hours post		0.705	1.959
	24 hours post		3.171	2.227
WvdW	Baseline		1.320	1.664
	4 hours post		0.511	2.021
	24 hours post		0.693	2.354

Antibody information

Activin receptor IIb

- **Manufacturer:** Abcam (anti-activin receptor IIB ab10596)
- **Specie:** goat polyclonal
- **Isotype:** IgG
- **Immunogen:** recombinant fragment (human)
- **Specie specificity:** human
- **Datasheet:**http://www.abcam.com/Activin-Receptor-TypeIIB-antibody-ab10596.thml

Smad6/7

- **Manufacturer:** Santa Cruz Biotechnology (Smad6/7 (N-19): sc-7004)
- **Specie:** Goat polyclonal
- **Isotype:** IgG
- **Immunogen:** N-terminal
- **Specie specificity:** mouse, rat and human
- **Datasheet:** http://www.scbt.com/datasheet-7004-smad6-7-n-19-antibody.html

Smad 2/3-p

- **Manufacturer:** Santa Cruz Biotechnology (p-Smad2/3 (Ser 423/425): sc-11769)
- **Specie:**rabbit polyclonal
- **Isotype:**IgG
- **Immunogen:** phosphorylatedSer 423 and Ser425
- **Specie specificity:** mouse, rate and human
- **Datasheet:** http://www.scbt.com/datasheet-11769-p-smad2-3-ser-423-425-antibody.html

Decorin

- **Manufacturer:** Abcam (Anti-decorin: ab54728)
- **Specie:** mouse monoclonal
- **Isotype:** IgG2b
- **Immunogen:** 1-360 aa human decorin
- **Specie specificity:** human and recombinant fragment
- **Datasheet:** http://www.abcam.com/decorin-antibody-ab54728.html

Myostatin

- **Manufacturer:** Abcam (anti-GDF-8: ab98337)
- **Specie:** rabbit polyclonal
- **Isotype:** IgG
- **Immunogen:** aa 300 – C-terminal
- **Specie specificity:** mouse, horse, chicken and human
- **Datasheet:** http://www.abcam.com/gdf8--myostatin-antibody-ab98337.html

Follistatin

- **Manufacturer:** Abcam (anti-follistatin: ab47941)
- **Specie:** rabbit polyclonal
- **Isotype:** IgG
- **Immunogen:** full length human follistatin
- **Specie specificity:** human
- **Datasheet:** http://www.abcam.com/follistatin-antibody-ab47941.html

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